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Description

This invention is concerned with the use of genetic engineering techniques in the modification of plants.

The invention provides chimeric genes, plasmid vectors, plants, plant seeds, plant tissue, cell cultur s and plasmids arising in and from the use of such genetic engineering techniques in the modification of plants, and methods and compositions for controlling or combating insects in agriculture or horticulture.

The invention is particularly directed towards introduction and integration of a chimeric gene coding for a polypeptide toxin produced by Bacillus thuringiensis or having substantial sequence homology to a toxin gene described below in plant cells and obtaining an insect controlling or combating level of expression of said polypeptide toxin intracellularly by transformed plant cells and their progeny.

Recombinant DNA technology is currently used to genetically engineer certain microorganisms such as bacteria and yeast to synthesize specific proteins. Genetic engineering of higher organisms within the present state of technology requires that one or a few cells be genetically engineered from which the entire organisms can develop. Among higher organisms, the cells of certain plants exhibit excellent regeneration capability and therefore are considered potentially good material for the genetic engineering of such plants. Furthermore, in higher plants, a known system is available to introduce foreign DNA into the plant genome. This system is provided by the tumor inducing plasmid from the gram negative soil bacterium Agrobacterium tumefaciens. Agrobacterium can genetically transform plant cells by stably integrating T-DNA, a well defined fragment of the Ti plasmid, into the plant cell genome. Recently, important progress has been made to facilitate the use of the Ti plasmid as a vector for plant genetic engineering. Small directly repeated sequences which flank the T-DNA (Border sequences) have been found to play a key role in the T-DNA integration. Nononcogenic Ti plasmid vectors have been constructed from which oncogenic tumor genes have been removed by an internal deletion in the T-DNA. These Ti plasmids still contain the border sequences and consequently transfer T-DNA without tumor induction. An example of such a Ti plasmid derived vector from plant genetic engineering is pGV3850 which contains a substitution of the internal T-DNA gene by the commonly used cloning vehicle pBR322. Several procedures have been developed to regenerate infected plants which contain the pGV3850. pGV3850 with the pBR322 sequences present in its T-DNA is an efficient acceptor plasmid for gene transfer experiments in plant cells. Indeed, genes cloned in pBR322 like plasmids are transferred to Agrobacterium and inserted via homologous recombination into the pGV3850 T-DNA in a single experimental step.

Another major advance in the development of plant engineering technique is the use of plant regulatory sequences to express chimeric genes in plants. In general, these chimeric genes contain a promoter region derived from a gene which is naturally expressed in plant cells, the sequence to be expressed, and preferentially a 3' non-translated region containing a polyadenylation site of a gene which is naturally expressed in plant cells. For example, using the nopaline synthase promoter and bacterial antibiotic resistance genes, dominant selectable markers for plant cells have been constructed.

Although certain chimeric genes have now successfully been expressed in transformed plant cells, such expression is by no means straightforward. Various lines of evidence indicate that the level of expression of the foreign genes of non-plant origin not only varies greatly in different transformed tissues but are in general very low. Such low levels of gene expression could be due to several reasons: first, incomplete transcription of the gene resulting from inadvertent transcription termination signals; second, inefficient processing of the messenger RNA; third, impaired transport of the messenger RNA from the nucleus to the cytoplasm; fourth, instability of the cytoplasm messenger RNA; fifth, inefficient translation of the cytoplasm messenger RNA; and sixth, instability of the protein due to its susceptibility to plant specific proteins. Consequently, the successful transformation of plant cells using vectors such as those described above is not necessarily predictable prior to attempting a desired transformation.

Engineering of differentiated plant cells and their progeny to express the Bt2 polypeptide and/or a truncated version thereof and/or a polypeptide having substantial sequence homology thereto is far more difficult than other genes such as antibiotic resistance genes or other plant genes such as thaumatin due to one or more of the following: (1) the large size of the Bt2 toxin, even in its truncated form; (2) the particular properties of the Bt2 polypeptide (such as, but not limited to, solubility of the polypeptide); (3) the potential toxicity of the Bt2 polypeptide toward the plant cells; or (4) the Bt2 polypeptide synthesized in plant cells and their progeny must retain substantially the same properties as the crystal protein synthesized in bacteria.

Bacillus thuringiensis (referred to at times herein as B.t.) bacteria includ s approximately 19 known varieties that produce polypeptide toxins which form parasporal crystals during sporulation. The crystal protein made by B.t. is toxic to the larvae of certain insects. The toxins produced by a particular variety exhibit strong insecticidal activity, against certain Lepidoptera and/or Coleoptera and/or Diptera larva. See

Also in accordance with the present invention there are provided: hybrid plasmid vectors comprising:
(a) a DNA fragment substantially homologous with that portion of a Ti plasmid essential for transfer of a T-region of a Ti plasmid to a plant cell genome (the virulence region of a Ti plasmid);
(b) at least one DNA fragment which delineates a DNA fragment to be integrated into a plant cell genome (the border sequences of the T-DNA portion of a Ti plasmid; where only one border sequence is present, preferably it is the right border sequence); and
(c) at least one chimeric gene comprising:
(i) a DNA fragment comprising a promotor region derived from a gene which is naturally expressed in a plant cell; and
(ii) at least one DNA fragment coding for a polypeptide toxin produced by Bacillus thuringiensis or at least one DNA fragment having substantial sequence homology thereto.

Said chimeric genes include those where DNA fragment (b) codes for a Bt2 protein, an insecticidally active truncated Bt2 protein, a DNA fragment having substantial sequence homology to Bt2 or the truncated Bt2, or where DNA fragment (b) is fused to a DNA fragment (c) coding for an enzyme capable of being expressed in differentiated plant cells and permitting identification of plant cells expressing DNA fragment (b) where said DNA fragments (b) and (c) express a fusion polypeptide.

Further, in accordance with the present invention, there are provided: intermediate plasmid vectors containing at least one chimeric gene, said chimeric gene comprising:
(a) a DNA fragment comprising a promotor region derived from a gene which is naturally expressed in a plant cell; and
(b) at least one DNA fragment coding for a polypeptide toxin produced by Bacillus thuringiensis, or at least one DNA fragment having substantial sequence homology thereto.

Said chimeric genes include those where DNA fragment (b) codes for a Bt2 protein, an insecticidally active truncated Bt2 protein, a DNA fragment having substantial sequence homology to Bt2 or the truncated Bt2, or where DNA fragment (b) is fused to a DNA fragment (c) coding for an enzyme capable of being expressed in differentiated plant cells and permitting identification of plant cells expressing DNA fragment (b) where said DNA fragments (b) and (c) express a fusion polypeptide.

Further, in accordance with the present invention, there are provided insecticidal compositions and methods of using transformed plant cells and their progeny.

Still further in accordance with the present invention are provided: a transformed plant cell containing a chimeric gene which: is stably integrated in the genome of the cell, is capable of being expressed in differentiated cells of a plant derived from the cell, and comprises:
(a) a promoter region derived from a gene which is naturally expressed in a plant cell; and
(b) a DNA fragment obtained by truncation of a DNA coding for a crystal protein produced by Bacillus thuringiensis or having substantial sequence homology thereto; the truncated fragment (b) coding for at least a polypeptide toxin of the crystal protein and providing an insect controlling amount of the polypeptide toxin in the cell as a result of intracellular expression of the truncated fragment (b);
plants which comprise such cells and can express the chimeric gene as described above; seeds which are capable of germinating into such plants and which can express the chimeric gene as described above; methods of protecting plants against a specific insect pest by transforming their genome with the chimeric gene as described above; and a method of transforming plants to protect them against a specific insect pest by integrating into their genome the chimeric gene as described above.

Transformed plant cells and their progeny intracellularly express a polypeptide toxin substantially similar to the polypeptide toxins produced by Bacillus thuringiensis and are substantially toxic to certain insects. Transformed plant cells and their progeny may be used in controlling insects.

Brief Description of the Drawings

Figure 1 is a photograph showing a 7.5% SDS PAGE stained with Coomassie Blue.
Track 1: B.t. kurstaki crystal protein preparation;
Track 2: B.t. berliner crystal protein preparation;
Track 3: Molecular weight markers
a: phosphorylase B (92,500 dalton);
b: bovine serum albumin (66,200 dalton);
c: ovalbumin (45,000 dalton); and
d: carbonic anhydrase (31,000 dalton).

Figure 2 is a schematic diagram of plasmid pEcoR251. The EcoRI endonuclease gene (EndRI) is fused to the P_R promotor (P_R) and contains a unique BglII cloning site. Amp: beta-lactamase gene.

Figure 19 shows the strategy used to construct Bt:NPTII fusions and Bt2 deletions.

Figure 20 is a schematic representation of the different Bt2 3' end deletion mutants, used in the mapping of the 3' end of the minimal toxin encoding fragment. Arrows repr sent the positions of the 3' ends.

Figure 21 is a photograph showing the results of an immunoblotting experiment using a rabbit anti-berliner crystal serum. Samples analyzed are total extracts from Bt2 deletion clones specified in Figure 20 and in Section 7.

Figure 22 shows the 3' end points of deletion clones pLB834 and pLB879 on the Bt2 sequence, used to delineate the minimal gene fragment encoding an active toxin. Also shown is the deduced amino acid sequence and the position of a putative trypsin cleavage site.

Figure 23 is a schematic representation of the construction of the Bt2:NPTII fusion gene cassettes pLBKm23, pLBKm33 and pLBKm14. Also represented are the 5' upstream sequences of the Bt2:NPTII fusions in the different constructs (sequences corresponding to a BamHI site are underlined).

Figure 24 is a schematic representation of different Bt:NPTII fusion gene cassettes.

Figure 25 is a photograph showing the results of a NPTII assay as described by Reiss et al. (Gene, 30, p. 217, 1984). The samples analyzed are the supernatants of cell extracts of bacterial clones producing NPTII or different Bt2-NPTII fusion proteins.

23 means K514 (lambda) (pLBKm23)

860 means K514 (lambda) (pLBKm860)

865 means K514 (lambda) (pLBKm865)

NPT means HB101 (lambda dv) (a gift from Julian Davis, formerly of Biogen)

Figure 26 shows the approximate positions of the 3' ends of the Bt sequences in different deletions and Bt:NPTII fusions (indicated by arrows).

Figure 27 shows the strategy used for the adaptation of the Bt2 and the Bt2:NPTII cassettes for expression in plant cells.

Figure 28 shows the DNA sequences at the junction between the promotor regions and the coding sequence of the Bt gene cassettes as they are present in the different engineered Ti plasmids. Sequences derived from the original promotor regions and from the coding sequence of the Bt2 gene are underlined. Some relevant restriction enzyme sites which have been involved in the assembly of the chimeric genes are indicated. The ATG initiation codon is boxed.

Figure 29 is a schematic representation of the construction of pHD208 as described in Section 8. Example 2.

B: BamHI, Hp: HpaI, H: HindIII, E: EcoRI, Bg: BglII.

Figure 30 is a schematic representation of the construction of pGV831: pGV831 has been constructed by R. Deblaere, Lab of Genetical Virology, Free University Brussels, Belgium. It is a derivative of pGV700, as described in European Patent Application No. 83112985.3. Recombinant DNA techniques used followed Maniatis et al., Molecular Cloning (1982), Cold Spring Harbor Laboratory.

The HindIII fragment present in pGV700 was subcloned into pGV600 (Leemans et al., J. Mol. Appl. Genet., 1, 149-164, 1981). Recombinant plasmid pGV742 was isolated as a Cb^R Cm^S Tc^S recombinant. An internal deletion was created in pGV742 by digestion with BamHI and recirculization. This produced pGV744. An internal deletion was created in pGV744 by digestion with EcoRI and recirculization to yield pGV749. The HindIII-NruI fragment from pGV749 was cloned in pGV710. pGV710 had been digested with EcoRI, the 5' protruding end filled in using DNA polymerase and had been subsequently digested with HindIII. The resulting plasmid pGV815 was isolated as a Sm^R, Cb^R recombinant. Both the EcoRI site and the HindIII site of pGV815 were removed by digestion with these enzymes and by filling in the protruding ends with DNA polymerase, followed by recirculization. Finally, a chimeric gene containing the nopaline synthase promotor and the neomycin phosfotransferase gene from Tn5 was isolated as a BclI-BamHI fragment from pKC7/nos and was cloned in the BglII site from pGV825. The Sp^R, Km^R recombinant plasmid pGV831 was obtained.

Figure 31 is a schematic representation of the T region of Ti-plasmid pGV3850 and of the intermediate vector pHD205. The crossed lines indicate the regions which were involved in cointegration of pGV3850 with pHD205 to produce pHD1050. The T region of hybrid Ti plasmid pHD1050 is represented.

H: HindIII

Bt: chimeric Bt2 gene under control of the nopaline synthase promotor

nos: nopaline synthase gene

Ap, Km: genes encoding ampicillin and kanamycin resistance

Figure 32 is a schematic representation of the T region of Ti plasmid pGV2260 and of the intermediate vector pHD208. The crossed lines indicate the regions which were involved in cointegration of pGV2260

exhibit similar properties.

"Identification" should be understood as referring to selection or scoring of cells harboring and expressing the desired gene. Selectable markers permit growth (selection) under otherwise lethal conditions such as kanamycin resistance (Km^R). Scorable markers add an identifiable trait (scoring) foreign to non-tranformed cells. "Naturally expressed gene" should be understood as meaning a DNA fragment whether originally part of a plant's genome or introduced by agents such as bacteria or viruses which produces RNA, protein or both in the plant in the absence of human intervention.

A chimeric gene may also include a nontranslated DNA fragment positioned on the 3' side (downstream) of the structural gene sequence, which in turn may include a regulatory signal referred to as a polyadenylation signal preferably derived from a gene which is naturally expressed in plants.

A naturally expressed gene includes a 3' non-translated region which in turn includes a polyadenylation signal, both of which code for the corresponding messenger RNA (mRNA) regions. These corresponding mRNA regions are located on the 3' side of a stop codon in a monocistronic mRNA. The 3' non-translated region of mRNA is believed to be involved in the processing, stability and/or transport of the mRNA. This 3' non-translated region of mRNA is also believed to contain a sequence of bases, polyadenylation signal, which is recognized by an enzyme in the cell. This enzyme adds a substantial number of adenosine residues to the mRNA molecule to form a poly-A "tail" on the mRNA.

Generally, the process used to arrive at the present invention is described in European Patent Application Publication No. 0116718 entitled "Process for the Introduction of Expressible Genes into Plant Cell Genomes and Agrobacterium Strains Carrying Hybrid Ti Plasmid Vectors Useful for this Process." The introduction and integration of one or more chimeric genes coding for polypeptide toxins produced by Bacillus thuringiensis or having substantial sequence homology to Bt2 (see Figure 13) into a plant cell genome is achieved by:

(1) isolation of at least one DNA fragment from Bacillus thuringiensis coding for a polypeptide toxin by digestion of bacterial DNA and inserting the mixture of DNA fragments obtained into a cloning vehicle harbored in a bacterial host; and

(2) identification of bacterial clones harboring DNA fragments coding for said polypeptide toxin; and

(3) characterization of the structure of the DNA fragment coding for said polypeptide toxin; and

(4) removal of unwanted DNA sequences flanking the desired DNA fragment; or

(5) synthesis of a DNA fragment having substantial sequence homology and exhibiting a similar structure to a DNA fragment coding for Bt2; or

(6) construction of a DNA fragment containing the DNA fragment from (4) fused to a DNA fragment encoding an identification polypeptide to produce a fusion polypeptide; and

(7) insertion of said DNA fragment from (4) or (5) or (6) into plasmid vectors under the control of plant regulator sequences harbored in a bacterial host; and

(8) introduction of plasmids from (7) by conjugation (or mobilization) in a bacterial host harboring suitable helper plasmids; and

(9) conjugation of bacterial clones from (8) to Agrobacterium tumefaciens harboring an acceptor Ti plasmid vector; and

(10) identification of Agrobacterium tumefaciens which contain the desired chimeric gene; and

(11) contacting plant cells with Agrobacterium tumefaciens from (10); and

(12) identification of transformed plant cells from appropriate culture media; and

(13) immunological detection of Bt2 antigens present in extracts from transformed plant cells; and

(14) propagating transformed plant cells to regenerate a differentiated plant.

It is contemplated that cloning vectors and bacterial host strains other than those described below in the Examples can be used. Ti-based vectors like pGV3850 into which recombinant plasmids integrate before transfer to plant cells are known as cis-type vectors. There are also Ti-based vector systems in which the recombinant plasmids do not integrate into the resident Ti plasmid or in which large portions of the naturally occurring Ti plasmid are deleted. These binary-type systems, Hoekema et al., Nature, Vol. 303, 179 (1983), or mini-Ti plasmids, Framond et al., Biotechnology, Vol. 1, 262 (1983), have also been shown to introduce DNA into plant cells. These plasmids contain a border sequence (at least one, preferably two) flanking the gene to be introduced into plants. A marker which is selectable or scorable in plant cells is useful but not essential. Such plasmids are capable of autonomous replication in A. tumefaciens and need not integrate into a resident Ti plasmid. Virulence functions needed to effect transfer to DNA, such as the chimeric genes of the present invention, to plant cells can be provided in trans. Hoekema et al., Nature, Vol. 303, 179 (1983). See also Fraley, R.T. et al., Biotechnology, Vol. 3, 629 (1985); and Klee et al., Biotechnology, Vol. 3, 637 (1985).

A. tumefaciens is not the only means of introducing genes into plants. DNA can be introduced by

A. Isolation of Bacillus thuringiensis (B.t.) crystal polypeptides
B. Preparation of antibodies (polyclonal and monoclonal) against B.t. crystal polypeptides
2. Preparations of B.t. Gene Bank
A. Preparation of total DNA or plasmid DNA from B.t., preferably plasmid DNA
B. Partial digestion of the purified DNA with a suitable restriction enzyme
C. Cloning DNA fragments into a suitable E. coli plasmid expression vector
3. Isolation of recombinant plasmids containing B.t. polypeptide genes
A. Screening of the transformed E. coli cells with anti-B.t. crystal protein serum
B. Identification and isolation of bacterial clones expressing the polypeptide
4. Characterization of Bt2 protein
A. Purification of the polypeptide encoded by the cloned B.t. gene
B. Testing to confirm that polypeptide expressed by clones is immunologically the same as B.t. crystal polypeptide
C. Testing to confirm that polypeptide expressed by clones is insecticidal
5. Mapping and subcloning of Bt2, including restriction enzyme analysis, subcloning and DNA sequence determination
6. Construction of toxin gene cassette including removal of undesired flanking ATG triplets preceding the initiator ATG and addition of suitable restriction enzyme cleavage sites using synthetic oligonucleotide linkers
7. Construction of Intermediate Vectors
8. Construction of Hybrid Ti Plasmids
9. Engineering of Plants
A. Identification of transformed plant tissues producing the toxin using the immunoassays and quantification of the toxin levels produced
B. Regeneration of plants from tissues
10. Detection of Bt2 toxin in engineered plants
11. Determine toxicity of engineered plants toward insects

Different types of chimeric genes (promotor-gene fusions), have been used to genetically transform plant cells, and basically 3 different types of plant specific promotors can be distinguished:

Promotors:

1. Ti plasmid derived promotors (Pnos, PTR at times referred to herein as PTR2)
2. Plant promotors (Pssu pea, Pssu301)
3. Plant virus promotors (P35S from cauliflower mozaic virus)

Types of chimeric genes:

1. Type I:

Straight promotor-gene fusions in which the entire Bt2 coding sequence is inserted behind the promotor fragment. Examples are: Pnos-Bt2 (pHD1050, pHD1060), Pssu pea-Bt2 (pHD1076), PTR2-Bt2 (pGS1161), Pssu301-Bt2 (pGS1181), P35S-1-Bt2 (pGS1261), P35S-2-Bt2 (pGS1271). Some of the constructs do not contain the intact 5' untranslated region of the original transcript (Pnos, Pssu pea), but others do (PTR, Pssu301).

2. Type II:

Chimeric Pssu-Tp-Bt2 gene fusion in which the Bt2 gene is fused to the transit peptide (Tp) sequence of the small subunit of RuBisco and expressed under the control of the Pssu promotor. In this case a fusion protein preferably is made from the natural translation initiation signal of the ssu gene. Van Den Broeck et al. (1985) demonstrated the transport of the bacterial NPTII protein into plant chloroplasts using a fusion between the transit peptide of the ssu of RuBisco and the NPTII coding region. In view of these results, we constructed the chimeric gene Pssu-Tp:Bt2. Both the Pssu promotor and the transit peptide (Tp) fragment were derived from the pea gene used by Van Den Broeck et al. (1985). Th DNA sequence at the junction site is shown in Figure 28. It is worth mentioning that the original 5' untranslated region of the pea m-RNA is maintained in Pssu-Tp:Bt2, so that the chimeric gene is translated from the genuine ssu translation initiation site (pHD1080).

3. Type III:

Straight promotor-gene fusions in which only part of the Bt2 coding sequence is used ("truncated Bt2"). Fragments of the Bt2 sequence still encoding an active toxin are inserted b hind the plant specific

and mice. Antiserum against B.t. crystal proteins (kurstaki) prepared in goat was received courtesy of Dr. L. Bulla, University of Idaho. To the best of applicant's knowledge and belief, the antiserum was prepared by known procedures substantially similar to those described for rabbit and mouse.

Rabbits were injected subcutaneously with 0.5 mg of a solubilized crystal protein preparation (.25 ml dialysed qagainst PBS pH 7.4) mixed with an equal volume of complete Freund's adjuvant (CFA). After three months, the rabbits received another injection of the same type of preparation, and three weeks later blood samples were taken. BALBc mice were injected intraperitoneally with 100 ug of crystal protein solution, mixed with CFA (1/1 vol.). Four to six weeks later they received a booster injection of 50 ug crystal protein PBS, and four days later blood samples were taken. Antigen reactivity of the sera was confirmed by immunodiffusion tests (Ouchterlony assay). A strong crossreaction between berliner 1715 and kurstaki crystal protein preparations was observed, indicating that they contained antigenically related components.

Some of the mice were sacrificed and the spleens removed asceptically for cell fusion experiments (see 2.2).

2.2 Monoclonal antibodies

Although not essential for the identification of toxin expressing clones as described herein, hybridomas producing monoclonal antibodies against B.t. crystal proteins were generated following the procedure originally described by Koehler and Milstein (Nature 256: 495-497, 1975). Monoclonal antibodies were used as an additional and more specific means of determining toxin presence in bacterial clones and plant cells.

Spleen cells from immunized BALBc mice (see 2.1) were fused with the SP2/0 myeloma cell line (Shulman, M. et al., Nature 276, p. 269, 1978). Cells were plated at 3.10^5 per well in microtiter plates and 10-14 days later the supernatants were screened for the presence of anti-crystal protein antibodies using an enzyme immuno assay (Engvall and Pesce, Scand. J. Immunol., suppl. 7, 1978) with alkaline phosphatase labelled goat anti-mouse immunoglobulin as the second antibocy (Sigma, A-5153). Approximately 4% of the wells were positive for the antigen (crystal protein). Positive clones were subcloned twice by limiting dilution. Positive subclones were selected, grown up and their culture supernatants containing the monoclonal antibodies were collected. A total number of 17 hybridoma cell lines producing monoclonal antibodies reactive with B.t. berliner crystal proteins were generated.

3. Construction of a gene bank from plasmid DNA of B.t. strain berliner 1715

Kronstad et al., J. Bacteriol., 54, p. 419-428 (1983) reported that B.t. berliner 1715 contains two related toxin genes which are both located on plasmids. Intact endotoxin genes were isolated from a gene bank from total B.t. berliner 1715 plasmid DNA using partial Sau3A digests of plasmid DNA. B.t. berliner 1715 cells were grown in LB medium (Miller, Experiments in Molecular Genetics, (1972), Cold Spring Harbor Laboratory, New York) overnight at 37° C. Plasmid DNA was isolated from B.t. berliner 1715 using the denaturation-renaturatien method described by Kronstad et al., J. Bacteriol., 54, p. 419-428 (1983). Analysis of the plasmid DNA on 0.5% agarose gels revealed that this plasmid DNA preparation contained several different plasmid species present in different molar concentrations. To construct the gene bank thirty ug of plasmid DNA was partially digested with Sau3A at 37° C in a total volume of 500 ul. 100 ul samples were taken after respectively 10, 20, 30, 45 and 60 minutes of incubation and phenol-chloroform extracted. The Sau3A digested DNA was size fractionated on a 10 to 40% sucrose gradient, and the size of the DNA fragments in the different fractions was estimated on a 0.8% agarose gel. The fractions containing DNA in the 6-10 Kb size range were pooled and ligated to BglII digested pEcoR251 vector DNA. The pEcoR251 plasmid is a derivative of plasmid pBR322 in which the EcoRI-PuvII fragment has been replaced by a chimeric EcoRI endonuclease gene which is fused to a P_R promotor fragment derived from plasmid pLK5 (Zabeau and Stanley, EMBO Journal, 1, 1217-1224 (1982)) as depicted in Figure 2. The pEcoR251 contains a unique BglII site in the EcoRI endonuclease gene, where insertion will inactivate the gene. The pEcoR251 vector is a suicide vector similar to the positive-selection cloning vehicle pSCC31 described by Cheng and Modrich (J. Bacteriol. 154, 1005-1008, 1983). Sau3A DNA fragments were ligated into BglII digested pEcoR251. Recombinant plasmids were selected by transforming the ligation mix into competent E. coli K514 cells (Colson et al., Genetics 52, p. 1043-1050, 1965) as described by Dagert and Ehrlich, Gene 6 (1980), 23-28. Cells were plated on LB medium (Miller, Experiments in Molecular Genetics (1972), Cold Spring Harbor Laboratory, New York), supplemented with ampicillin (100 ug/ml).

Several gene banks were constructed each containing between 600 and 1500 recombinant clones. Analysis of the recombinant plasmids present in 12 randomly chosen clones confirmed that in each gene bank at least 10 out of the 12 clones contained inserted fragments with sizes ranging from 5 to 15 Kb.

obtained from a 2 litre overnight culture of K514 (pBt200) were resuspended in 50 ml 50 mM TRIS pH 7.9, 50 mM EDTA, 15% sucrose, treated with lysozyme (100 ug/ml), sonicated (30 minutes at 400 watts in a Labsonic 1510), mixed with 200 ml of PBS, pH 7 containing 2% Triton X100 and incubated for 30 min. on ice. The lysate was centrifuged at 15000 g and the supernatant was discarded. The pellet containing the Bt2 protein was resuspended in the same buffer and the procedure was repeated. Whereafter the pellet was washed twice with 200 ml PBS. To solubilize the Bt2 protein the pellet was resuspended in 50 ml extraction buffer 0.2 N thioglycolate and 0.1 M $NaHCO_3$, pH 9.5 for 2 hr. at 37° C. An efficient (>90%) and selective solubilization of Bt2 protein was obtained in this way (Figure 7).

These semi-purified protein preparations were used for further studies. Antisera were raised against Bt2 protein in rabbits and mice using a similar immunization protocol as described in Section 2.1. These antisera reacted equally well with solubilized crystal proteins from B.t. berliner and kurstaki as with Bt2 itself, in the ELISA assay described above (Figure 8 shows results with the mouse serum).

A similar positive reaction was observed using antibodies purified, from anti-Bt crystal serum, by affinity chromatography on an immunoadsorbent of Bt2 (Bt2 protein coupled onto CNBr activated Sepharose 4B, Pharmacia). These antibodies also reacted in Western blotting with a 130Kd protein present in both B.t. berliner and kurstaki crystals.

Finally, in the ELISA, 9 out of the 17 monoclonal antibodies raised against total B.t. berliner crystal proteins, were also reactive with the Bt2 protein. (Code numbers: 1F6, 167, 4D6, 4F3, 8G10, 10E3, 1.7, 4.8, C73) (Figure 9). The same 9 antibodies were also reactive with B.t. kurstaki crystal proteins.

In general both the Bt2 protein and the major 130 Kd crystal proteins from B.t. require alkaline pH and the presence of reducing reagents for complete solubilization. Also they both precipitate at pH 4-5.

Thus, the cloned gene product Bt2 exhibits biochemical properties similar to those of the major 130 Kd crystal protein from B.t. berliner and B.t. kurstaki and is immunologically related to these crystal proteins.

The Bt2 protein was purified further by DEAE-ion exchange chromatography and by Sephacryl gel filtration. The amino-terminal sequence of this purified protein was determined with the use of a gas-phase sequencer (Applied Biosystems), operated according to Hewick et al., J. Biol. Chem., 256, 7990-7997, 1981).

The sequence of the first 20 N-terminal amino acids was found to be substantially identical to the N-terminal sequence deduced from the DNA sequence of a cloned B.t. kurstaki gene. Wong et al., J. Biol. Chem., 258 (3), 1960-1967 (1983) (Figure 10).

5.2 Insect Toxicity of the Bt2 protein

Crystals from B.t. are known to be particularly toxic against larvae of certain Lepidoptera species. In order to test whether Bt2 protein exhibited a similar toxic activity, toxicity tests were performed on larvae of the cabbage butterfly Pieris brassicae. Protein solutions of known concentration, expressed as ppm (1 ppm = 1 ug/ml) were serially diluted in water. Small discs (0.25 cm^2) were cut from fresh cabbage leaves and on each disc 5 ul of a test solution was applied. Discs were air dried and each disc was placed in a vial containing one larva. Third instar larvae were obtained from a synchronized culture of P. brassicae. During a 10 h period before moulting, these larvae were incubated in separate vials in the absence of food. Immediately after moulting they were given one leaf disc. When the first disc was consumed, the larva was offered a fresh disc without sample. For each sample dilution, 50 larvae were tested. Feeding and viability were monitored every 24 h up to 120 h. As can be seen from Table 1, Bt2 sample preparations exhibited similar degrees of toxicity for P. brassicae larvae as solubilized crystals from B.t. berliner 1715.

To test the effect of sublethal doses of Bt2 toxin on the growth of P. brassicae larvae, the following experimental design was used: cabbage leaves were dipped in a solution containing a known concentration of Bt2 protein (0.01-1 ppm) and dried. Groups of 100 third instar larvae (from synchronized cultures) were fed on Bt2 coated leaves. The leaves were regularly replaced by new leaves treated in the same way. Growth of the larvae was followed over a period of seven days, which corresponds to the time period needed to develop from 3rd to 5th instar. As can be seen from the results presented in Table 2 the Bt2 protein induced a significant growth inhibition in P. brassicae larvae at doses that were sublethal. Growth inhibition was evident at a concentration of 0.01 ppm which corresponded to 2.67 ng protein/gram leaf. During the first 48 h the larvae feeding on leaves coated with 0.01 ppm ate 3.6 cm^2 of leaf (83 mg) and consequently ingested about 0.22 ng of Bt2 protein. At this time, 93% of the larvae were still in the L3 stag while only 33% of the control larvae were in this stage. Thus an inhibitory effect on growth can be observed with toxin doses that are significantly below the LD_{50} values (1.65 ng/larva, see Table 1).

These results indicate which levels of Bt2 prot in synthesis must be reached in transformed plant cells in order to express insect resistance against P. brassicae. A level of 2.7 ng Bt2 protein/g tissue is sufficient

BamHI for 1 h at 37° C. Subsequently, 50 ng DNA was recircularized with 0.1 unit T4 DNA ligase in a total volume of 100 ul for 20 h at 4° C.

One-fifth of this ligation mixture was transformed into competent E. coli K514 cells (Colson et al., Genetics 52 (1965), 1043-1050) as described by Dagert and Ehrlich, Gene 6 (1980), 23-28. Cells were plated on LB medium (Miller, Experiments in Molecular Genetics, (1972), Cold Spring Harbor Laboratory, New York), supplemented with carbenicillin (100 ug/ml).

The deletion end points in the plasmids were first analyzed by measuring the size of the newly generated EcoRI fragments of the recombinant plasmids on a 2% agarose gel. The nucleotide sequences of the exact deletion end points in plasmids with deletions ending just before the start of the Bt2 gene were determined. Clone pHD100 has a deletion ending 8 bp before the initiator ATG and removes all upstream non-initiator ATG's. Clone pBa3.3 contains the BamHI linker fused to the 4th bp of the coding sequence and clone pBa23-3 contains the Bam linker fused to bp -33.

In a second engineering step, the non-coding sequences at the 3' end of the toxin gene were deleted using Bal31 exonuclease (Biolabs, New England). Thirty ug of pHD100 plasmid DNA were digested with NdeI and treated with Bal31 exonuclease for 3, 4, 5, 6 and 8 minutes at 30° C in buffer. At each time interval, 60 ul aliquots (each containing 6 ug of Bal31 treated DNA molecules) were removed. After addition of phosphorylated BglII linkers (Biolabs, New England) to the Bal31 treated DNA molecules, the DNA molecules were recircularized with 0.1 U T4 ligase overnight at 4° C. The ligation mixture was transformed into competent E. coli K514 cells (Colson et al., Genetics 52 (1965), 1043-1050) as described by Dagert and Ehrlich, Gene 6 (1980), 23-28. Cells were plated on LB medium (Miller, Experiments in Molecular Genetics, (1973), Cold Spring Harbor Laboratory, New York) supplemented with carbenicillin (100 ug/ml). After determination of the size of the deletion in several plasmids, using restriction enzyme digestion and agarose gel electrophoresis, pHD160, pHD162, pHD163 were retained for further experiments. In pHD160, the BglII site is positioned at approximately 300 bp behind the TAA stopcodon of the Bt2 gene; in pHD162 the BglII is at approximately 250 bp behind TAA; and in pHD163 the BglII is at position 3342 (bp) in the Bt2 coding sequence. Construction of pHD160 is schematically diagrammed in Figure 15. In this way, we constructed toxin gene cassettes carrying the Bt2 gene on a BamHI-BglII fragment which will be excised and inserted in the BamHI site of the Ti expression vectors. In order to construct pHD164, the BamHI-SacI fragment of pHD160 containing the 5' end of the coding sequence was replaced with the corresponding BamHI-SacI fragment of pBa3.3. To construct pHD159, the BamHI-SacI fragment of pHD163 was replaced by the BamHI-SacI fragment of pBa3.3 (Figure 16).

In order to create plasmid pDC3 (Figure 16), plasmid pHD164 was digested with DraI, ligated to BglII linkers, and the fragment containing the Bt2 gene was cloned in the BglII site of pLK57 (Figure 17). In this way, the BglII site of the BamHI-BglII cassette was placed in close proximity of the TAA stop codon of Bt2.

7.2 Construction of cassettes containing engineered Bt2 genes

7.2.1 Truncated Bt2 genes

7.2.1.1 Rational

Results from basic research on the functional properties of B.t. crystal proteins indicate that the large approximately 130 Kd crystal proteins are relatively insoluble and, in addition, are protoxins which need processing in the insect midgut towards lower molecular weight active toxins, able to exert their toxic effects on the insects (Bulla, L. A., Jr., D. B. Bechtel, K. J. Kramer, Y. I. Shetna, A. I. Aronson and P. C. Fitz-James, 1980, Rev. Microbiol., 8:147-203; Bulla, L. A., Jr., K. J. Kramer, D. J. Cox, B. L. Jones, L. I. Davidson and G. L. Lookhart, 1981, Biol. Chem., 256:3000-3004; T. A. Angus, Can. J. Microbiol., 2:416 (1956); M. M. Lecadet, "Microbial Toxins", Vol. II, ed. by T. C. Montie and S. Kadis, Academic Press, Inc., New York and London, 1970, pp. 437-471). The specific activity of the Bt toxin when ingested by the insects as part of a composition of engineered plant material will be determined, not only by the total quantity of toxin present but also by the degree of accessibility of active toxin, released in the midgut. It has been shown that some insects species are more efficient than others in solubilizing and/or "processing" (enzymatically degrade) B.t. protoxins (Presentation by Dr. P. Luthy in "Second Worksnop Bacterial Protein Toxins", Wepion, Belgium: June 30-July 4, 1985; to be published in congress proceedings). Therefore, it might be advantageous in the engineering of insect resistant plants to construct truncated toxins derived from Bt2 which have the properties of being: 1) already processed or partially processed toxin, exhibiting full toxic activity; and 2) more soluble than the original Bt2 protein. Plants expressing such truncated polypeptides might exhibit a higher specific toxicity against insects than plants excressing intact Bt2 at the same level.

7.2.2 Fusion genes to NPTII

7.2.2.1 Rationale

It is known that amino-terminal fusions at the NPTII gene can generate fusion proteins that still confer kanamycin resistance in bacteria (Reiss et al., EMBO J. 3, p. 3317, 1984).

Since NPTII is a most suitable selection marker in plant engineering, such gene fusions could have very promising applications. Indeed when using such NPTII fusion proteins to transform plants, a selection for high kanamycin resistance would allow direct selection for a high expression of the fusion product. Therefore, toxin gene fusions with NPTII might be used to transform plants and select for transformed plants expressing high levels of toxin, by selection for kanamycin resistance.

7.2.2.2 Construction of the fusion gene cassettes

Different fragments of the Bt2 gene were fused to the N-terminus of NPTII.
One of the fusion proteins termed Bt:NPT2 is described in more detail below.

1. Construction of the Bt:NPT2 fusion gene

The construction of the Bt:NPT2 gene is shown in Figure 23. pLK54 is a pBR322 derivative containing the P_L promotor and 2 phage fd transcription terminators in tandem (Section 7.2.1.2). pKm109/90 contains the NPTII gene of Tn5 on pBR322 (Reiss et al., EMBO J., 1984) (Figure 24).

A 1141 bp gene fragment of pKm109/90 containing the NPTII gene was cloned in pLK54 giving rise to pLKm90. In order to create a BglII site behind the NPTII gene, BglII linkers were ligated at the XbaII and the SalI site after Klenow polymerase treatment. This gives rise to pLKm91.

pHD159 is a derivative of pBt200 (Section 7.1) whereby a BamHI linker has been fused to the 4th bp and a BglII linker to bp 3342 (after Bal31 treatment). The BamHI BglII fragment of this plasmid containing the deleted Bt2 gene was inserted in the BamHI site of pLKm91, in one orientation, giving rise to a Bt2:NPTII fusion gene on pLBKm10.

To construct pLBKm13 an Asp 728, Klenow treated BglII fragment was inserted between the BamHI site (after filling in) and the BglII site of pLKm91.

In order to produce the Bt:NPTII fusion proteins in E. coli, analogous constructs to pLBKm10 and 13 were made containing 5' leader sequences of the Bt2 gene with a ribosome binding site. Therefore, from another Bal31 deletion derivative of pBt200, pBa23-3 (Section 7.1), with the BamHI linker at position -33 we exchanged the BamHI-SacI fragment with pLBKm13, giving rise to pLBKm23.

For the expression of the fusion protein Bt:NPT2 behind the Pnos promotor and the 35S promotor, the BamHI-SacI fragment of pHD160 (described in Section 7.1) was cloned between the same sites in pLBKm13 giving pLBKm33.

Finally for the construction with the Petunia ssu-promotor (see Section 8) we used a modified Bt:NPTII cassette wherein the 3' non-coding region was removed up to the stopcodon of NPTII. To achieve this the NCo1-BglII fragment of pLBKm13 containing the 3' end of the NPTII gene was replaced by a NCo1 BglII-fragment generated from pLKm91 (Figure 23). This plasmid was cut with DdeI, treated with Klenow polymerase, and ligated to a BglII linker, whereafter the resulting DNA was cut by NCo1 and BglII. Figure 23 also shows the 5' Bt2 sequences in the different constructs.

In summary, the Bt:NPT2 gene contains (Figure 24):

1) The 5' end of the Bt2 gene starting 8 bp upstream of the initiation ATG codon or at pos +4 or at position -33 and extending towards nucleotide position 2173.
2) a 16 bp linker fragment.
3) the NPTII coding region starting at nucleotide position 13.

2. Characteristics of the fusion protein expressed in E. coli

The fusion gene Bt:NPT2, placed behind the P_L promotor in plasmid construction pLBKm23 (Figure 23), was expressed in E. coli to study the properties of the fusion protein.

2.1 Identification of the fusion protein in E. coli

An E. coli clone transformed with pLBKm23 was analyzed in SDS-PAGE and in W stem blotting.

intact Bt2 (Tables 4 and 5).

Taken together the above data indicat that the Bt:NPT2 protein has NPTII activity both "in vivo" and "in vitro" and in addition that it has an equally potent insecticidal activity as the Bt2 toxin. Therefore, this truncated toxin clearly represents a valuable alternative in the engineering of plants expressing high level of insect toxicity.

2.5 Selection of additional Bt2:NPTII fusions expressing a higher kanamycin resistance phenotype

The results obtained with the previously constructed Bt:NPTII gene were very promising since this fusion protein conferred kanamycin resistance, showed normal levels of toxicity and was relatively stable. However, the kanamycin resistance conferred by the Bt:NPT2 fusion was relatively low as compared to the wild type cells. Since the isolated purified Pnos-Bt:NPT2 protein had a specific NPTII activity comparable to the wild type NPTII protein, we concluded that the low kanamycin resistance was due to the low solubility of the fusion protein in E. coli. A relatively low resistance phenotype might interfere with an efficient selection system in plants. Therefore, we considered the possibility that other Bt-NPTII fusions could have different physicochemical properties leading to a higher Km resistance phenotype "in vivo". We designed an experiment to fuse the NPTII gene at random to the Bt2 sequence in the region between the KpnI site and the processing site. The experiment was as follows:

We used plasmid pLBKm25 (Figure 18) containing the following elements of interest: a P_L promotor and the Bt2 gene, fused at 1100 bp downstream of the KpnI site, to the NPTII gene (see Figure 19). A unique XhoI site separates the Bt from the NPTII gene. This plasmid was linearized by KpnI digestion and treated with Bal31 exonuclease. The Bal31 reaction was titrated such that the deletions did not proceed far beyond the HindIII site which is localized upstream of the C-terminal processing site. The Bal31 treated plasmid was ligated to XhoI linkers, digested with XhoI and self-ligated. As a result, the NPTII is fused to fragments of the Bt2 gene varying in size. These plasmids, transformed in E. coli, conferred kanamycin resistance on condition the NPTII gene was fused in frame to the Bt2 gene. Transformants were selected on plates containing low levels of kanamycin (20 ug/ml) and screened for the ability to grow on higher kanamycin concentrations.

145 kanamycin resistant transformants were screened for their ability to grow on higher kanamycin concentrations. 8 transformants proved more resistant and were able to grow on concentrations higher than 200 ug/ml of kanamycin. The fusion point in all 8 clones was determined by restriction enzyme mapping with an accuracy of 20 bp. Surprisingly 7 out of 8 clones had their fusion point around the HindIII site at position 1680 of the Bt gene. One clone (pLBKm860) mapped at position approximately 2050. Although the majority of the deletions were fused around position 1800, none of these conferred a higher kanamycin resistant phenotype. The 7 clones which have their fusion point positioned around the HindIII site are too short to encode an active toxin. However, one of the clones (pLBKm860) was:

More stable, since more protein per amount of total cellular extract was detected in Western blot analysis; and

More soluble since more truncated Bt protein was detected in the supernatant.

The positions of the 3' end points in the Bt2 coding secuences in clones 860 and 865 are represented in Figure 26.

Toxicity of the fusion proteins and truncated Bt2 gene products is illustrated in Table 6.

7.3 Adaption of cassettes containing truncated Bt genes or Bt gene fusions for expression in plant cells

Plasmids pLBKm860 and 865 were modified as described in Figure 27 to generated plasmids pLBKm1860 and pLBKm1865 respectively. pLBKm2860 was derived from (Figure 27) pLBKm860.

By replacing the BamHI-SacI fragment from pLB820 and 884 for the BamHI-SacI fragment of pLBKm14, the new plasmids called pLB1820 and 1884 respectively, were generated. pLB2820 was derived from pLB1820. As an examole, the final constructs pLBKm1860, pLBKm1865 and pLBKm2860 are shown in Figure 24.

8. Construction of intermediate expression vectors containing the toxin gene

8.1 Overview

Table 7 gives an overview of the engineered plasmids which have been constructed and used in the plant tranformation experiments. Each engineered Ti plasmid is th result of a cointegration of a receptor Ti

sequence of the Bt gene cassettes as they are present in the different engineered Ti plasmids. Sequences derived from the original promotor regions and from the coding sequence of the Bt2 gene are underlined. Some relevant restriction enzyme sites which have been involved in the assembly of the chimeric genes are indicated. The ATG initiation codon is boxed.

Junctions between Bt gene cassettes and the 3' ends

All chimeric genes are provided at the 3' end with a sequence which contains the 3' untranslated region, including the polyadenylation site, of a gene which is naturally expressed in plant cells. The following sequences have been used:

3'ocs (in pHD1060, pHD1076, pHD1080)

A 706 bp PvuII fragment containing the 3' untranslated region of the octopine synthase gene (pos 11939-11233 according to Gielen et al., EMBO 4, p. 835, 1984).

3't7 (pGS1151, pGS1161, pGS1152, pGS1153, pGS1162, pGS1163, pGS1251, pGS1261, pGS1253, pGS1262, pGS1271, pGS1281

A 212 bp EcoRV-ClaI fragment containing the 3' untranslated region of T-DNA gene 7, cloned into the SmaI site of pUC8 and reisolated as a EcoRI-SalI fragment (pos 2317-2105 according to Gielen et al., EMBO 4, p. 835, 1984).

3'nos (in pGS1110)

A 182 bp Ta9I-ClaI fragment, containing the 3' untranslated region of the nopaline synthase gene (pos 1290-1472 according to Depicker et al., J.M.A.G. 1, p. 561, 1982)

3'SSu301 (in pGS1171, pGS1181)

A approximately 1.2 Kb BglII-BamHI fragment derived from the 3' end of the ssu301 gene was constructed by site-directed mutagenesis as follows:

```
                    stop coding region (which is TAA)
ssu301              ...TTCTAAGTTATA
coding              ...TTCTAAGATCTATA
sequence
                                        Construction of a BglII
BglII                                   site through site-
                                        directed mutagenesis
```

Example 8.1

This example describes the construction of pHD205, an intermediate vector containing a chimeric Bt2 toxin gene comprising: the nopaline synthase promotor, the Bt2 toxin gene cassette from pHD160 and a DNA fragment containing the 3' untranslated region of the nopaline synthase gene including the polyadenylation site. In the chimeric gene the Bt2 gene cassette is oriented such that the expression of the Bt2 protein can be obtained from the nopaline synthase promotor. The Bt2 gene cassette was excised from pHD160 with BamHI and BglII and inserted in the BamHI site of pLGV2382 (Herrera-Estrella et al., EMBO J., 2, 987-995, 1983). Two ug of pHD160 DNA were totally digested with respectively 2 units of BglII and BamHI (Boehringer Mannheim) for 1 h at 37° C in a final volume of 20 ul, using the incubation buffer described by Maniatis et al. (Molecular Cloning (1982), Cold Spring Harbor Laboratory, 133-134). Five ug of pLGV2382 DNA was totally digested with BamHI under the same conditions. Subsequently the terminal 5' phosphates were removed from the DNA by treatment with calf intestinal alkaline phosphatase (CIP)

HindIII-BamHI fragment containing the Km^R gene of pKC7 was substituted by the 20 bp HindIII-GamHI polyiinker of pUC8.

Five ug of pGV858 were digested with 5 units of BamHI for 1 h at 37° C in a final volum of 20 ul, using the incubation buffer described by Maniatis et al., Molecular Cloning, Cold Spring Harbor Laboratory, 133-134, (1982). Subsequently, the terminal 5' phosphates were removed from the DNA by treatment with CIP using the conditions described by Maniatis et al., Molecular Cloning, Cold Spring Harbor Laboratory, 133-134 (1982). Two ug of pGV861 were digested with 2 units of BglII, BamHI and PvuI for 1 h at 37° C in a final volume of 20 ul, using the incubation buffer described by Maniatis et al., Molecular Cloning, Cold Spring Harbor Laboratories 1982.

0.2 ug BamHI digested and CIP treated pGV858 was ligated to 0.05 ug BamHI-BglII-PvuI digested pGV861 with 0.01 units of T4 DNA ligase (Boehringer Mannheim) in a final volume of 20 ul. The ligation mixture was transformed into competent E. coli K514 cells (Colson et al., Genetics 52 (1965), 1043-1050) according to Dagert and Ehrlich, Gene, 6 (1980), 23-28. Cells are plated on LB medium (Miller, Experiments in Molecular Genetics (1972) Cold Spring Harbor Laboratory, New York) supplemented with carbenicillin (100 ug/ml). Carbenicillin resistant clones were screened for the presence of recombinant plasmids by restriction enzyme digestion of DNA prepared by the microscale technique described by Birnboim and Doly (Nucl. Acids. Res. 7 (1979), 1513-1523).

In one of the recombinant plasmids, pHD503, the BglII-BamHI fragment including the pea ssu promotor is inserted in the correct orientation in front of the 3' end of the octopine synthase gene. pHD503 contains a unique BamHI site, located between the Pssu promotor and the 3' end of the octopine synthase gene.

Step 3: Insertion of the BamHI-BglII Bt2 gene cassette into the BamHI site of pHD503 to yield the intermediate expression vector pHD208. Two ug of pHD160 DNA were completely digested with 2 units of BglII and 2 units of BamHI for 1 hour at 37° C in a final volume of 20 ul. Five ug of pHD503 DNA were digested with 5 units of BamHI to completion under the same conditions, treated with CIP using the conditions described by Maniatis et al., Molecular Cloning (1982), Cold Spring Harbor Laboratory, 133-134) to remove the terminal 5' phosphates from the DNA. 0.1 ug of BamHI-BglII digested pHD160 DNA was ligated to 0.2 ug of BamHI digested and CIP treated pHD503 DNA with 0.01 U T4 DNA ligase in a final volume of 20 ul.

The ligation mixture was transformed into competent E. coli K514 cells (Dagert and Ehlich, Gene 6 (1980) 23-18). Cells were plated on LB medium (Miller, Experiments in Molecular Genetics (1972), Cold Spring Harbor Laboratory, New York) supplemented with streptomycin (20 ug/ml) and spectinomycin (50 mg/ml). Streptomycin-spectinomycin resistant clones were screened for the presence of recombinant plasmids by restriction enzyme digestion of DNA prepared from these clones by the microscale technique described by Birnboim and Doly (Nucl. Acids Res. 7, 1513-1523, 1979). pHD208, a recombinant plasmid containing the Bt2 gene cassette in the correct orientation with respect to the Pssu promotor was isolated and used in further experiments.

Example 8.3

This example describes the construction of pGSH151. The intermediate vector pGSH151 contains a chimeric Bt:NPTII fusion gene comprising: the promotor of transcript 2 of the TR-DNA of the octopine Ti plasmid (PTR2) (Velten et al., 1984, Embo J., 3, 2723), the Bt:NPTII fusion gene cassette from pLBKm13 and the 3' untranslated region of the gene 7 of the T-DNA of the octopine Ti plasmid.

The fragments of the chimeric gene were assembled as described in this example. All the techniques were performed as described in Maniatis et al., Molecular Cloning (1982).

Step 1: Construction of pGSH50 (Figure 41)

This plasmid contains the TR promotor PTR2 with a completely intact 5' untranslated region, followed by an ATG-initiation cocon, followed by a unique BamHI site, and the 3' untranslatec end of the transcript 7 gene.

pOP443 (Velten et al., 1984) contains a ClaI-HdIII fragm nt comprising the PTR2 and the PTR1 of the octopine Ti plasmid. To eliminate the BamHI site, pOP443 was totally dig sted with BamHI and SalI, th sticky ends treated with the Klenow fragment of E. coli polymerase I and self-ligated with T4-ligase.

After transformation, ampicillin-resistant colonies were selected and their plasmids were screened for the absence of BamHI and SalI sites, yielding pOP4433SF.

In order to create a ClaI site in front of the 3' untranslated end of transcript 7 in pAP2034 (Velten et al., 1984), pAP2034 was totally digested with BamHI, treated with the Klenow fragment of E. coli polymeras I

rifampicin (100 ug/ml) and kanamycin (25 ug/ml). The physical structure of the T region of one of the transconjugants, pHD1050, was determined according to the method described by Dhaese et al., (Nucl. Acids Res. 7 (1979), 1837-1849) by hybridization of P^{32} labelled pHD205 against HindIII digested to total DNA of C58Cl Ri f^R pHD1050. Th structure of the T region of pHD1050 is diagrammed in Figure 31.

Example 9.2:

The intermediate expression vector pHD208 was inserted into the acceptor Ti plasmid pGV2260 to yield the hybrid Ti plasmid pHD1076. As diagrammed in Figure 32 pHD1076 contains the chimeric Bt2 gene under the control of the Pssu promotor as well as a chimeric gene containing the neomycin phosphotransferase gene under the control of the Pnos promotor, positioned between T-DNA border fragments. The Ti plasmid pGV2260 is described in European Patent Application Number 83112985.3 (Publication Number 0116718). The plasmid pHD208 was introduced into competent E. coli GJ23 cells by transformation according to Dagert and Ehrlich (Gene 6 (1980), 23-28). To select for E. coli GJ23 cells transformed with pHD208, the transformation mixture was plated on LB medium (Miller, Experiments in Molecular Genetics - (1972), Cold spring Harbor Laboratory, New York) supplemented with carbenicillin (100 ug/ml).

Liquid LB medium was inoculated by one of the transformed E. coli colonies and cultured overnight. 0.1 ml of the overnight culture of the E. coli strain carrying all 3 plasmids was conjugated overnight with an overnight culture of the C58Cl Rif^R (pGV2260) at 28 °C on LB medium (Miller, Experiments in Molecular Genetics (1972), Cold Spring Harbor Laboratory, New York). Agrobacterium strains containing hybrid Ti plasmid, resulting from a single cross-over event between pGV2260 and pHD208 were isolated by selecting for the streptomycin-spectinomycin marker carried by the pHD208 plasmid on minimal A medium (Miller, Experiments in Molecular Genetics (1972), Cold Spring Harbor Laboratory, New York) supplemented with spectinomycin (300 ug/ml) and streptomycin (300 ug/ml) and streptomycin (1 ug/ml).

Transconjugants were purified on LB medium (Miller, Experiments in Molecular Genetics (1972), Cold Spring Harbor Laboratory, New York) supplemented with rifampicin (100 ug/ml), spectinomycin (100 ug/ml) and streptomycin (300 ug/ml). The physical structure of one of the transconjugants, pHD1076, was determined by hybridizing P^{32} labelled pHD208 against PstI digested total DNA of C58Cl Rif^R pHD1076 according to the method described by Dhaese et al., (Nucl. Acids Res. 7 (1979), 1837-1849). The physical structure of pHD1076 is shown in Figure 32.

Example 9.3

The intermediate expression vector pGSH151 was inserted into the acceptor Ti plasmid pGV2260 to yield the hybrid Ti plasmid pGS1151.

The method used was a triparental cross according to Dittag et al. (1980), PNAS, 77, 7347-7351.

Liquid LB medium was inoculated with one of the pGSH151 transformed E. coli K514 colonies and cultured overnight at 37 °C. 0.1 ml of this culture was plated together with 0.1 ml of overnight cultures of HB101 (pRK2013) Figurski & Helinski (1979), PNAS, 76, 1648-1652 and 0.1 ml of C58Cl Rif^R (Van Larebeke et al., Nature, 252, 169-170) on LB plates and grown overnight at 28 °C.

The cells were collected from the LB plates and dilutions were plated on minimal A. medium (Miller, Experiments in Molecular Genetics, 1972, Cold Spring Harbor Laboratory, New York) supplemented with spectinomycin (300 ug/ml) and streptomycin (1 mg/ml). Transconjugants were purified on LB medium containing rifampicin (100 ug/ml), spectinomycin (100 ug/ml) and streptomycin (300 ug/ml). The physical structure of one of the transconjugants, pGS1151, was determined by hybridizing P^{32} labeled pGSH151 against PstI-BamHI digested total DNA of C58Cl Rif^R (pGS1151) according to Dhaese et al., N.A.R., 7 (1979) 1837-1849.

10. Isolation of plant cells and plants containing the chimeric toxin gene inserted in their genome

Procedures:

Two different protocols are described here for the transformation of tobacco plant cells with transformation vectors such as those described in Section 9 and for the generation of callus tissue and/or differentiated plants from these transformed cells.

Procedure 1: Cocultivation of protoplasts

a) Grow nopaline positive or kanamycin resistant calli for 4 weeks.
b) Transfer the differentiating calli on hormone free Murashige and Skoog.
c) Grow for 3 weeks.
d) Separate shoots and transfer to the same medium, grow for 2-3 weeks till plants form roots.
e) At this stage small plants are transferred to grow in 250 ml containers containing 50 ml of half strength hormone free Murashige and Skoog medium.
f) Grow for 2-3 weeks. Remove a lower leaf for nopaline detection or screening of kanamycin resistance activity and for immunological detection of the toxin.

The leaf disc (also at times referred to herein as leaf segments) assay for testing Km resistance of a plant is performed as follows. Small discs are cut out from "in vitro" grown plants and transferred to petri dishes containing callus inducing medium (M&S macro and micronutrients and vitamins 3% sucrose, 500 mg/l Claforan, 1 mg/l NAA and 0.1 mg/l BAP) with various kanamycin sulphate concentrations (50-500 mg/l).

After three weeks incubation in a plant tissue culture room, callus growth on the leaf discs is monitored. The Km resistance level of the plant is determined as the highest concentration of Km on which the leaf discs still give rise to callus tissue.

Screening for the presence of nopaline (nopaline assay) is performed according to the procedures described in Aerts M., Jacobs M., Hernalsteens J-P., Van Montagu M. and Schell J. (1979) Plant Sci. Letters 17, 43-50.

Composition of medium 55:

- Half strength of the Macronutrients of the Murashige and Skoog salts
- 1 ml/l of 1000 x Micronutrients Heller modifed
- 1 ml/l of 1000 x vitamins Morel & Wetmore
- 100 ml/l Inositol
- 10 ml/l of a stock solution containing $FeSO_4$ 5.57 g/l and Na_2EDTA 7.45 g/l
- Benzylaminopurine 1 ml/l
  Naphthalene acetic acid 3 mg/l
- Mannitol 80 g/l (0.44M)

Sucrose 20 g/l

1000 x Vitamins Morel and Wetmore for 100 ml

Ca pantotenate 100 mg;
Biotine 1 mg;
Niacine 100 mg;
Pyridoxine 100 mg;
Thiamine 100 mg;

Micronutrients Heller modified (500 ml)

500 mg $ZnSO_4 . 7H_2O$
50 mg H_3BO_3;
50 mg $MnSO_4 . 4H_2O$
50 mg $CuSO_4 . 5H_2O$
15 mg $AlCl_3$;
15 mg $NiCl_2$

Composition of medium 56:

Medium 56 is the same as medium 55 except for the addition of naphthalene acetic acid at 0.2 mg/l and glutamine 1 mM.

Procedure 2: Infection of leaf segments with Agrobacterium strain C581 Rif^R containing a hybrid Ti plasmid

This procedure describes the infection of leaf segments with C58C1 Rif^R and the isolation of transformed cell lines by selection on kanamycin containing medium.

Sterile Nicotiana tabacum cv. Petite Havana SR-1 plants were grown in vitro in plant nutrient agar containing half strength of the complete Murashige & Skoog (M&S) salt mixture complemented with half strength of the organic nutrients and sucrose of complete M&S medium. Twenty SR-1 leaf segments of

T-DNA: Pssu-Bt2 (Bt2 gene fused to Pssu)
Selectable marker: kanamycin resistance
Transformation method: leaf disc infection.

Using conditions described in procedure 2 either callus transformation of shoot induction was performed on the infected leaf discs. Using the callus induction protocol, a number of calli were obtained by partial purification and maintained as separated semi clones. On the basis of positive immunoassay results 5 of these lines were selected for further propogation (1076-4, 10, 11, 12, 13). From the shoot induction protocol used in the initial stage of leaf disc infection a number (72) of kanamycin resistant plants were regenerated (selection on 50 ug/ml Km).

When retested by leaf disc assay 65% of these proved to be truly resistant to 50 ug/ml Km. From leaves of some "in vitro" propagated plants, callus tissue was generated and propagated "in vitro" for further testing.

Example 10.4: Calli and plants transformed with pHD1080

T-DNA: Pssu - Transit peptide (Tp) Bt2
Selectable marker: kanamycin resistance/(Nos)
Transformation method: leaf disc infection.

Kanamycin resistant calli and shoot were induced following procedure 2. Approximately 20 kanamycin resistant callus lines were analyzed for nopaline expression and all were found positive. 86 kanamycin resistant shoots were selected, propagated "in vitro" and retested for kanamycin resistance (using the leaf disc assay) and for nopaline expression.

52 plants (60%) were both kanamycin resistant and nopaline positive, and these were further propagated "in vitro." Approximately 10% of the plants expressed only one of the two markers.

Example 10.5: Plants transformed with pGS1110

T-DNA: Pnos-Bt:NPTII (fusion)
Selectable marker: kanamycin resistance/Nos
Tranformation method: leaf disc infection.

Leaf discs from "in vitro" maintained SR-1 plants were incubated during 48 hours with a suspension of Agrobacterium tumefaciens C58Cl Rif^R pGS1110 (procedure 2). Similar dilutions of different control strains containing chimeric genes encoding intact NPTII were included. After two weeks active shoot formation on M&S medium containing 50 mg/l kanamycin was observed both with the controls and pGS1110. However, after transfer to fresh selective M&S medium, a difference became apparent between the controls and pGS1110. Some shoots on discs inoculated with the latter strain turned yellow and were growing slowly. The best growing and green shoots were transferred to medium without kanamycin. Part of them could be rescued in this way and started growing normally after the second transfer on kanamycin free medium.

About 70 shoots were rescued from the pGS1110 transformation experiment. Screening among 35 of these shoots showed that 28 of these (85%) were real transformants since they produced nopaline. This important observation suggests that, although the shoots have not been maintained for a long period on Km containing medium, phenotypical selection for the expression of the fusion protein had occurred.

The obtained shoots were propagated "in vitro" as small plants on nonselective medium. A number of these plants were tested for Km^R resistance using the leaf disc assay. Most of them expressed a certain level of Km^R since they formed callus on Km containing medium. Variable resistance levels were recorded in the range of 50-500 mg Km/liter. However, most of the plants were only resistant to low levels of Km. Two out of a total of 61 plants showed resistance to 200 ug/ml Km and partial resistance to 500 ug/ml Km (very weak callus growth).

For a number of plants, copies were transferred into vermiculite pots. When reaching 10-15 cm h ight a first insect toxicity test was performed on leaves of these plants (s e section 13).

Example 10.6: Plants transformed with pGS1161

T-DNA: PTR2-Bt2
Selectable marker: kanamycin resistance

the ELISA described in Section 5 and adapted for assaying plant extracts.

Conditions for preparing and assaying plant extracts were established in reconstruction experiments in which purified Bt2 protein was mixed with plant extracts.

In reconstruction experiments we observed no significant loss in antigenic activity of Bt2 protein (less than 20%) due to the presence of plant extracts. In the ELISA assay, as little as 0.1 mg/ml purified Bt2 protein was still detectable. However, in reconstruction experiments a certain variability in background occurs, probably caused by plant proteins present in extracts. Therefore, reliable detection limit in these conditions was of the order of 1 ng/g tissue, which corresponds to a level of 2 ng Bt2 protein per g of plant tissue.

11.1 Screening of individual calli

For the immunological screening of individual calli, the following experimental procedure was established:

Two hundred mg of callus tissue was mixed with 150-200 ul of extraction buffer. Extraction buffer had the following composition: 50% of a solution of Na_2CO_3 500 mM and DIT 100 mM and 50% fetal calf serum. The tissue was homogenized by crunching with a spatula whereafter the cell debris were centrifuged. Fifty ul of supernatants was added to 50 ul of PBS pH 7.4 + 10% fetal calf serum in wells of a microtiter plate coated with goat antibodies against B.t. crystal protein as described. During the entire procedure the samples were kept in ice and the microtiter plates were incubated at 4° C for 1.5 - 2 hours. Thereafter the ELISA procedure was continued as described in 5.1 for detection of Bt2 protein with either rabbit anti-Bt2 serum or with a mixture of monoclonal anti-Bt2 antibodies 4D6, 10E3, 1.7, and 4.8 (under the form of culture supernatants).

Example 11.1:
Analysis of calli transformed with C58Cl Rif^R pHD1050.

Transformed callus clones were obtained through the protoplast cocultivation method as described in Section 10 Example 10.1. Since 19% of the clones were found to express nopaline (Nos^+), at least 19% of them were transformed. However, due to an additional border sequence in the intermediate expression vector (pLGV2382) the nos gene and the Bt2 gene can be inserted independently as well as tandemly. Therefore both Nos^+ and Nos^- clones were screened in the ELISA assay.

A total of 180 callus clones (130 nos^-, 50 nos^+) were tested. Some of the clones were retested once or twice at different time intervals after the initial propagation from protoplast culture. In none of the cases could a clear positive signal be recorded. When the substrate reaction times of the assay were prolonged (overnight incubation at 4° C) some of the clones (both nos^+ and nos^-) produced a very weak signal above the background (background being control callus without Bt2 gene). However, since the obtained values were clearly below the reliable detection limit of the test system, no firm conclusions could be drawn concerning the expression of Bt2 protein in these calli.

Example 11.2:
Detection of Bt2 protein in tobacco callus tissue transformed with C58Cl Rif^R pHD1076.

Transformed callus tissue obtained from leaf segment infections using Agrobacterium strain C58Cl Rif^R (pHD1076) (see Section 10, Example 10.3), were screened immunologically for the presence of Bt2 protein.

After initial propagation calli were transferred for a second time after 20 days. When they reached optimal growth, 200 mg was used from each callus line for immunological screening in the ELISA. In a first experiment 9 out of 14 transformed calli showed a positive signal clearly above the background obtained with the 4 control calli (untransformed SR-1 callus), when reacted with a specific rabbit anti Bt2 serum. (see Figure 34). Three transformed calli generated a signal corresponding to approximately 5 ng Bt2 protein per gram tissue, as determined by comparison with a positive control (control SR-1 mixed with a known amount of Bt2 protein). All samples gave signals equal to background level signals (obtained with SR-1 control callus) when reacted with normal rabbit serum as a negative control. In a second experiment 13 out of 21 transformed calli yielded a signal significantly above background (Figure 35). One of the calli generated a signal corresponding to 4 ng of Bt2 per gram tissue. These results indicate that Bt2 protein is produced at a detectable level in a fraction of the calli transformed with pHD1076.

About 5 weeks after the first ELISA experiments, 4 selected lines (1076-10, 11, 12 and 13) which in th initial screening gave high positive values, wer retested in ELISA. From each lin several "subclones"

extract (total volume 2 ml). Reconstruction experiments (Bt2 added to SR-1 control callus at the beginning of the extraction), indicated that only 20% of Bt2 protein is lost during the extraction procedure and that 80% is contained in the pH 4.5 fraction. Based on these results, one could calculate that the total amount of Bt2 protein originally present in 140 g callus was 305 ng, which is 2.2 ng/g, a result that agrees well with the original estimates made for the screening of individual calli (see Figures 34 and 35). These data show that Bt2 protein present in extracts from transformed calli can be specifically concentrated using a precipitation procedure at pH 4.5 as described above, allowing us to quantify more accurately the amount of Bt2 protein produced in these plant tissues.

Example 11.2.2: Calli transformed with pHD1050.

A 500 g pool of selected callus clones (on the basis of previous ELISA tests on individual calli, approximately 25 callus lines, which gave values above background, were selected) and homogenized in the presence of 1000 ml extraction buffer using the same procedure as described in Example 1. Material which remained soluble at pH 6, but precipitated at pH 4.5 was isolated by centrifugation and subsequently redissolved in a small volume of carbonate buffer pH 10 (see Example 11.2.1). Analysis of the material in ELISA revealed positive signals corresponding to 60 ng/ml Bt2 or 1.2 ng/g callus tissue (Table 8).

Example 11.2.3: Calli transformed with pHD1060 and pHD1080.

In this example a slightly different and more extensive extraction protocol was used for the isolation of Bt2 protein from the engineered plant material. A protocol was developed to recover eventual residual Bt2 protein that would not be solubilized in a single extraction step as used in the procedures of Examples 11.2.1 and 11.2.2. Such could be the case, since Bt2 protein contains some highly hydrophobic regions which possibly interact with plant cell membrane structures and therefore would be difficult to solubilize in the absence of detergents. The step by step procedure used here would allow the recovery of additional proteins associated with insoluble plant cell structure. A schematic representative of the protocol is given in Figure 37.

A first protein fraction is obtained by extraction in carbonate buffer pH 10 + DTT and concentration through acid precipitation (pH 4.5) (fraction I). This fraction corresponds to the pH 4.5 extract obtained using the procedure in Examples 11.2.1 and 11.2.2.

Material not solubilized in this first extraction step and remaining in the pellet is then treated with the same extraction buffer containing 1% Triton X-100. Proteins, solubilizing in these conditions and precipitating at pH 4.5 are contained in fraction II. The last step involves solubilization in 2% SDS followed by acetone precipitation, yielding fraction III. Fractions I and II are analysed in ELISA and Western blotting; fraction III, which contains SDS, is only analysed in Western blotting.

ELISA results are given in Table 8: positive signals were detected in fractions I and II of both constructions 1060 and 1080, corresponding to Bt2 levels of respectively 1.9 and 1.4 ng/g original tissue (fr. I) and 0.27 and 0.29 ng/g (fr. II). Western blotting of the SDS solubilized material (fraction III) revealed the presence of a faint approximately 130 Kd band for both 1060 and 1080 callus material (using rabbit anti-Bt2 serum). Detection limit of the Western blotting was 10 ng/lane, therefore these fractions contained at least 0.39 ng/g for 1060 and 0.5 ng/g for 1080.

Western blotting of fractions I of 1050, 1060 and 1080 did not reveal the presence of a 130 Kd band probably because the concentration of Bt2 protein is too low in these fractions.

The present results indicate that low levels of Bt2 protein are indeed expressed in calli transformed with pHD1060 and pHD1080. Although small scale analysis of individual calli might not allow detection of immunopositive clones in these constructions, a more rigorous extraction and concentration procedure on a pool of selected calli clearly results in reliable and quantitative detection of Bt2 protein. A substantial fraction of the Bt2 was strongly bound to insoluble plant material and could only be released upon use of detergents such as Triton and SDS.

11.3 Detection of Bt2 protein in leaves of regenerated transformed plants

For the routine testing of leaf samples the following procedure was established:

Green leaf tissue (200-400 mg) was taken from "in vitro" grown plants (5-10 cm high as described in Section 10, Example 1) and homogenized in the presence of extraction buffer (200 ul), containing 50% of: Na_2CO_3 500 mM, 100mM DTT, 480 ug/ml leupeptine (Sigma, L-2884), 2 mM PMSF, 2mg/ml ascorbic acid (Sigma, A-7631), 2 mM EDTA and 50% of FCS. Th tissu was homogenized by crunching with a spatula

Procedures:

Toxicity assays were performed on first instar larvae of Manduca sexta, fed on artificial diet. Three to four ml of liquid artificial diet (Bell, R.A. & Joachim, F.G. (1976) Ann. Entomol. Soc. Ann. 69: 365-373) were dispensed in each compartment (4 cm^2) of square Petri dishes. Formaldehyde was omitted from the diet. After the diet had become solid, 200 ul of a known dilution of sample was applied on the surface of the diet and dried in a cool air flow. Four newly hatched larvae were placed into each compartment. Growth and mortality were followed over a period of 3-5 days.

Example 12.1: Callus extract from calli transformed with pHD1076.

A concentrated extract from a pool of calli, transformed with pHD1076 was prepared as described in Section 11.2 Example 11.2.1. Extract dilutions were applied onto the surface of the diet and its toxicity was evaluated. The extracted material 1076 pH 4.5 clearly had a toxic effect on the Manduca sexta larvae: at 12.5 ul/cm^2 all larvae showed growth inhibition and at 50 ul/cm^2 100% died (Table 11). Toxic activity of this material was significantly diminished after immunoprecipitation (100% normal growth at 12.5 and 25 ul/cm^2 and only 37% death at 50 ul/cm^2), indicating that the toxic activity can be depleted by anti-Bt2 antibodies. Extract from untransformed SR-1 callus tissue, the negative control, was completely nontoxic. Since the presence of Bt2 protein in the extracts was quantified immonologically, we could correlate the observed toxicity with the determined Bt2 conce ntration. The immunoassay values indicated that 1076 pH 4 contained 122 ng/ml Bt2. Thus, 50 ul extract per cm^2 corresponds to 6.1 ng Bt2 protein/cm^2. Previous toxicity assays with Bt2 on Manduca (Section 5.2, Table 3) indicated that Bt2 at 12 ng/cm^2 is 100% lethal while 2.5 ng/cm^2 induces growth inhibition.

Together these results indicate that the Bt2, expressed in engineered callus tissue, is a functional toxin and displays toxicity which is in the same range of potency as the bacterial Bt2 gene product.

13. Insecticidal activity exhibited by leaves of transformed tobacco plants

Procedures:

In order to evaluate the insecticidal activity expressed in leaves of transformed tobacco plants the growth rate and mortality of Manduca sexta larvae feeding on these leaves was recorded and compared with the growth rate of larvae feeding on untransformed SR-1 leaves. The following procedures were used:

Procedure 1

Subsequent experiments were carried out on leaf discs placed in Petri dishes. Four leaf discs of 4 cm diameter were punched out, placed on wet filter paper on a Petri dish together with 4 x 10 first instar larvae of M. sexta. Preferentially young leaves from the upper part of the plant were used. Twenty-four hours later a second disc was added. Between 48 hours and 100 hours after initiation of the experiment, the number of moulted insects were counted at regular time intervals. From this we could calculate the MT_{50} time at which 50% of the larvae had moulted. The whole experiment was conducted in a growth chamber at 26° C, 90% relative humidity and under a photoperiod of 16 hours light and 8 hours darkness.

In order to estimate the toxin levels required to have a notable effect on growth rate and viability of Manduca sexta larvae in the present experiment, a series of reconstruction experiments had to be included. To this end purified solubilized Bt2 protein (Section 5.1) was serially diluted in PBS containing 0.5% Triton X-100. Standard volumes of Bt2 solution were mechanically sprayed (to obtain a very homogenous coating) on tobacco leaf discs. Ten L_1 (first instar) larvae were placed on each leaf disc, and 3 discs were used per Bt2 concentration. Growth rate and mortality of the larvae were followed over a 100 hour period.

Procedure 2

A procedure essentially similar to the previous one was also used. This experimental protocol was however somewhat more extensive in order to be more effective in reliably detecting very small effects on larval growth rate. The set up was different from the previous one in the following aspects:

- care was taken that all plants were in exactly the same stage and condition so that the effects on larval growth caused by differences in the condition of the leaf tissue would be minimal.
- larval growth was followed up to the L_3 stage (unlik previous experiments where growth was only

were retested in Experiment II, in order to confirm the observed toxicity effects.

Experiment I:

The test was performed as described in Procedure 2 (this section) except that only two groups of ten larvae were used per plant (newly hatched Manduca sexta larvae).

Growth rate and mortality of the larvae were followed over a 7 day period and the larval weight at the end of this period was determined. Detailed results from Experiment I are represented in Table 15 and indicate that larvae feeding on several plants transformed with pGS1151 show significant growth inhibition in the initial stage of the experiment, as compared to larvae feeding on a control plant. For example, after 71 h, 60% of the larvae feeding on control plant N21-107 have gone to the L2 stage, while the number of L2 larvae is only 15% or less on plants N21-18, 43, 53, 50 and 11. When followed over a longer period, significant mortality was recorded in the larvae feeding on pGS1151 transformed plants. On one of the plants (N21-11), mortality reached 100% after less than 7 days. Mortality on the control plant only reached 15% on day 7 and 45% of the larvae had already gone to the L3 stage (this in contrast to the other plants having substantially no L3 larvae on day 7).

Experiment II:

Results from a second insect test (II) involving newly hatched M. sexta larvae was performed on some of the plants also used in Exp. I, following Procedure 1. Results are presented in Table 16. A high mortality rate was recorded in the plants transformed with pGS1151 (75-100% death) while nearly all the larvae feeding on the control plants N21-102, 104 and 107 were still viable after 4 days. A complete list of all the plants used in insect tests I and II is given in Table 17. Also indicated are the Km resistance levels determined for the plants transformed with pGS1151; the percentage mortality of the larvae feeding on these plants after several days; and the mean weight of the larvae that survived after 7 days in Experiment I.

Conclusion:

Tobacco plants transformed with pGS1151 and selected for high Km resistance clearly induce severe toxic effects on larvae feeding on these plants. The effects on insect larvae observed here, are the same as those induced by the B.t. toxin of bacterial origin (see Section 5.2, Tables 2 and 3); that is, growth inhibition in the initial stage (retardation in the transition from one instar to the next) followed by death.

It is apparent from Table 17 that the plants exhibiting the highest levels of Km resistance (500 ug/ml Km) also induce the highest mortality rates. Thus, using the fusion protein construction, we were able to select for efficient expression of toxicity by selecting for Km resistance.

It should be noted that the use of a fusion protein, as described herein, may represent a particular advantage, not only because direct selection for transformants of interest can be done, but also because the fusion protein itself might have some intrinsic useful properties. For example, Bt2:NPTII fusion proteins might be more stable in plant cells than intact Bt2 protein and/or the messenger RNA derived from the fusion genes might be more stable than intact Bt2 RNA.

14. Stable inheritance of new phenotype, acquired through transformation

A substantial fraction of the plants transformed with the transformation vectors described herein will contain, stably inserted into their genome, a fragment of newly acquired DNA containing both a chimeric Bt toxin gene and a marker gene (nos, NPTII). This was confirmed by the results of southern blotting experiments. The new phenotypic traits acquired through this transformation method (expression of Bt toxin, antibiotic resistance, nopaline production) will be inherited according to classic Mendelian genetics. To verify stable inheritance of the new traits, F_1 descendants from transformed plants were analysed for the expression of Bt toxin and synthesis of nopaline.

Transformed tobacco plants were allowed to flower and give seed. Care was taken that no cross pollination occured. From 4 plants previously identified as Bt^- (161-9, 10^{-1}, 147^{-3}, 174), seeds were germinated in agar medium and F_1 plants were analysed for the presence of nopaline (nopaline synthase being present as marker gene in the parental plants). Plants were tested 3 weeks after germination (approximately 1 cm in height) or later at 6-7 weeks (2-4 cm). The results are depicted in Table 18.

From plants 10-1 and 147-3 about 3/4 of the F_1 were nos^+, which is expected from Mendelian

E. coli K514 (pLBKm1820)	ATCC 53388
E. coli JM83 K12 (pSSU301)	ATCC 53391
E. coli K514 (pLBKm1860)	ATCC 53387
A. tumefaciens C58C1 Ery^R Cml^R (pHD1080)	ATCC 53385
A. tumefaciens C58C1 Rif^R (pGS1110)	ATCC 53386
A. tumefaciens C58C1 Rif^R (pGS1151)	ATCC 53392
A. tumefaciens C58C1 Rif^R (pGS1161)	ATCC 53393
A. tumefaciens C58C1 Rif^R (pGS1152)	ATCC 53394
A. tumefaciens C58C1 Rif^R (pGS1163)	ATCC 53395
A. tumefaciens C58C1 Rif^R (pGS1171)	ATCC 53396
A. tumefaciens C58C1 Rif^R (pGS1181)	ATCC 53397
A. tumefaciens C58C1 Rif^R (pGS1182)	ATCC 53398
A. tumefaciens C58C1 Rif^R (pGS1251)	ATCC 53399
A. tumefaciens C58C1 Rif^R (pGS1261)	ATCC 53400
A. tumefaciens C58C1 Rif^R (pGS1253)	ATCC 53401
A. tumefaciens C58C1 Rif^R (pGS1262)	ATCC 53402

Their viability was tested and confirmed on 27th December 1985.
Cultures of E. coli K514 are commercially available

TABLE 2

Effect of Bt2 Protein on Growth Kinetics of P. brassicae Larvae
(Results Expressed in % of Larvae in a Certain Stage); 1 ppm = 267 ng/gram leaf

Bt2 Concentration

	0						0.01 ppm						0.1 ppm					
Time (hours)	Stage L3	WC	L4	WC	L5	% mort	L3	WC	L4	WC	L5	% mort	L3	WC	L4	WC	L5	% mort
24	100					0	100					0	100					0
48	33	67				0	93	7				0	100					-
52	16	50	34			0	70	30				0	100					-
57		30	70			0	55	45				0	100					-
71	3	97				0	44	15	41			0	100					60
77		100				0	15	18	67			0	100					-
95		100				0	3	5	92			0	100					85
102		89	11			0	3	5	92			0	100					85
119		63	30	7		0		3	97			0	100					95
127		36	40	24		0			97	3		0						100
143		7	58	35		0			45	51	4	0						
151		6	22	72		0			24	70	6	0						
167				100		0			15	27	58	0						

TABLE 4

Toxicity of Bt:NPT2 Fusion Protein on 3rd Instar P. brassicae (% Mortality After 4 Days)

	Toxin dose (ug/ml)				
Bt protein	0.1	0.2	0.3	0.6	1
Bt2	70	NT(x)	90	NT	100
Bt:NPT2	NT	80	NT	100	NT

(x) NT = Not Tested

TABLE 6

Toxicity of Bt:NPTII Fusion Proteins or Bt2 Deletions on 3rd Instar P. brassicae Larvae (% Mortality After 4 Days)

E. coli strain	Exp. 1	Dilution 1/100	Bacterial Extract 1/10	1/3
NF_1 (neg. control)		0	0	0
pLBKm860		100	98	100
pLBKm865		2	0	0
	Exp. 2	1/25	1/5	1/1
NF_1		14	2	2
pLB879		100	100	100
pLB834		2	2	0
	Exp. 3	1/100	1/10	1/1
NF_1		4	4	2
pLB879		8	50	98
pLB820		54	100	100
pLB884		74	100	100

TABLE 8

Results Immunoassays on Pooled Callus Extracts

Construction	Extract Fraction	Protein Content ug/ml	Total Volume Extract (ml)	Bt2 in ELISA ng/ml	ng/g	Western Blotting Volume (ul)	130 Kd
pHD1050 (500 g)	I	9650	10	60	1.2	50	-
pHD1060 (392 g)	I	7800	8	95	1.9	50	-
	II	640	1	105	0.27	200	±
	III	N.D. (x)	0.3	N.D.	N.D.	20	+
pHD1080 (100 g)	I	4150	2	72	1.2	50	-
	II	326	1	29	0.29	N.D.	N.D.
	III	N.D.	0.5	N.D.	N.D.	100	+

(x) N.D. = Not Determined

TABLE 9

Levels of Bt2 Protein Detected in Leaves from 5 Immunopositive Plants Transformed by pHD1050	
Plant Isolation Number	ng Bt2/g Plant Tissue
161-9	25.0
10-1	7.6
10-2	6.0
147-8	14.0
147-9	9.2

TABLE 11

Toxicity of Callus Extract on Manduca Sexta Larvae

Extract	Volume Per cm^2 (ul)	Total Number Larvae	Results After L1	WC	L2	Dead
1076	12.5	4	3	1		
pH 4.5	50	4				4
	100	4				4
SR-1 pH 4.5	50	8			8	
(Control No Plant Extract)		44		1	43	
After Immunoprec:						
1076	25	12			12	
pH 4.5	50	8		1	3	4
SR-1 pH 4.5	50	8			8	

TABLE 13

Growth Rate of Manduca sexta Larvae Feeding on Tobacco Leaves from Plants Transformed with pGS1110

Exp. 1: Number of Larvae in a Certain Stage After 87 h:

Plant No:	C1	C2	C3	N20-3	N20-46	N20-38	N20-22	N20-47	N20-18	N20-30	N20-31
Stage L1	6	0	3	7	5	15	12	4	14	6	0
L2	14	20	17	13	14	4	6	16	6	13	20
Dead	0	0	0	0	1	1	2	0	0	1	0

Exp. 2: Number of Larvae in a Certain Stage after 78 h:

Plant No:	C4	C5	C6	C7	N20-35	N20-37	N20-7(*)	N20-7(*)	N20-19	N20-13	N20-1
Stage L1	0	0	0	1	1	11	1	0	0	1	0
L2	20	20	20	19	19	9	19	20	20	19	20
Dead	0	0	0	0	0	0	0	0	0	0	0

(*) Two copies of this plant were tested in this experiment.

TABLE 15

Growth Rate and Mortality of Manduca sexta Larvae Feeding on Leaves From Tobacco Plants Transformed with pGS1151 (Experiment I)

Represented are:

Numbers of larvae in a certain stage (L1, L2 or L3) or dead (D) from groups of 20 larvae after a period of feeding on the tobacco leaves.

Time	Plant N21-50				N21-35				N21-11				N21-56			
(Hours)	D	L1	L2	L3	D	L1	L2	L3	D	L1	L2	L3	D	L1	L2	L3
0		20				20				20				20		
55		20			5	15				20				20		
61	1	19			5	14	1		1	19				20		
66	1	19			5	11	4		1	19				19		
71	1	19			6	5	9		3	19			1	9	10	
76	1	18	1		7	4	9		5	15			1	8	11	
81	1	18	1		7	4	9		5	15			2	7	11	
87	1	18	1		7	4	9		5	15			2	7	11	
92	2	17	1		8	3	9		8	12			2	3	15	
119	11	7	2		12	1	7		18	2			3	1	16	
136	12	4	4		12	8			19	1			4		16	
144	12	4	4		15	5			19	1			4		16	
159	13	3	4		17	3			20				4		16	
168	15	1	4		17	2	1		20				4		15	1

TABLE 16

Growth Rate and Mortality of Manduca sexta Larvae Feeding on Leaves from Tobacco Plants Transformed with pGS1151 (Experiment II) (See also Legend for Table 15)

Time	N21-50		N21-18		N21-43		N21-11		N21-56		N21-35		N21-53		N21-33		Controls N21-102			N21-104			N21-107		
(hours)	D	L1	D	L1	D	L1	D	L1	D	L1	D	L1	D	L1	D	L1	D	L1	L2	D	L1	L2	D	L1	L2
0		20		20		20		20		20		20		20		20	20			20			20		
29												20		20		20	20			20			20		
47											5	15	6	14	8	12	20			20			20		
51	9	11	8	12	2	18	18	2	2	18															
57											8	12	15	5	15	5	20			20			20		
69	16	4	16	4	10	10	20		3	17															
79	19	1	18	2	11	9	20		3	17	16	4	18	2	20		14	6		15	5		20		
96											17	3	20		20		4	16		12	8	1	18	1	
100	19	1	18	2	14	6	20		10	10															
118	20		19	1	18	2	20		15	5															
120											18	2	20		20			20			20	1	13	6	

*Plants N21-102, 104, 107 are control plants transformed with PTR:NPTII.

TABLE 17 (cont'd)

Plant No.	Km^R (ug/ml Km)	% Mortality Exp. I (after 168 h)	Exp. II (after 118 h) (or 120 h*)	Mean Weight Surviving Larvae (mg/larva) Exp. I (after 168 h)
45	200	30	N.T.	13.7
50	500	75	100	10.7
53	500	90	100*	12.5
56	200	20	75	22.4
Controls:				
N21-102	—	N.T.	0*	N.T.
104	—	N.T.	0*	N.T.
107	—	15	5*	44.1

N.T. = Not Tested

TABLE 18

Frequency of Nopaline Positive Plants in the F_1 Generation Derived from Transformed Tobacco Plants

Plant No. of Parental Plant	Age of the Seedlings Tested (wks)	Total Number of Plants Tested	Nopaline Positive	% Nopaline Positives
147-3	3	74	56	76%
	7	13	11	85%
10-1	3	25	20	80%
	7	9	7	78%
161-9	3	66	18(x)	27%
	7	107	81	76%
174	6	45	43	95%

(x) Nopaline Signal Very Weak.

Claims

1. A transformed plant cell containing a chimeric gene which is stably integrated in the genome of said cell, is capable of being expressed in differentiated cells of a plant derived from said cell, and

fragment (c) codes for an enzyme capable of being expressed in said differentiated plant cells and permitting identification of plant cells expressing said truncated fragment (b).

18. The cell as claimed in claim 17, wherein said marker fragment (c) provides for kanamycin resistance or encodes neomycin phosphotransferase.

19. The cell as claimed in any of claims 1 to 18, wherein said promoter is a nopaline synthase promoter (**Pnos**), a small subunit of ribulose phosphate carboxylase promoter (**Pssu**), such as **Pssu301**, a Ti plasmid-derived promoter (**PTR**), or a promoter fragment derived from cauliflower mosaic virus (**P35S-1**) or (**P35S-2**).

20. A plant or differentiated plant cell progeny which comprises the plant cell as claimed in any of claims 1 to 19.

21. A seed of a plant or progeny thereof as claimed in claim 20.

22. A method of protecting a plant against a specific insect pest which comprises transforming the genome of said plant with said chimeric gene as claimed in any of claims 1 to 19 whereby an insect controlling amount of said polypeptide toxin is expressed in cells of said plant.

23. A method of transforming a plant to protect it against a specific insect pest which comprises stably integrating, in the genome of said plant, said chimeric gene as claimed in any of claims 1 to 19 whereby an insect controlling amount of said polypeptide toxin is expressed in cells of said plant.

Revendications

1. Cellule végétale transformée, contenant un gène chimérique qui : est intégré de façon stable dans le génome de ladite cellule, est capable d'être exprimé dans des cellules différenciées d'une plante issue de ladite cellule, et comprend :
   (a) une région promotrice issue d'un gène qui est naturellement exprimé dans une cellule végétale ; et
   (b) un fragment d'ADN obtenu par troncature d'un ADN codant pour une protéine cristal produite par Bacillus thuringiensis ou ayant une séquence substantielle homologue à celle-ci ; ledit fragment tronqué (b) codant pour au moins une toxine polypeptidique de ladite protéine cristal et fournissant une quantité luttant contre les insectes de ladite toxine polypeptidique dans ladite cellule comme résultat de l'expression intracellulaire dudit fragment tronqué (b).

2. Cellule selon la revendication 1, dans laquelle ledit fragment tronqué (b) est adjacent ou fusionné à un fragment (c) marqueur d'ADN, pouvant être sélectionné ou quantifié, dans ledit gène chimérique.

3. Cellule selon la revendication 2, dans laquelle ledit fragment marqueur (c) est fusionné audit fragment tronqué (b) de sorte que lesdits fragments (b) et (c) codent pour un polypeptide de fusion.

4. Cellule selon l'une quelconque des revendications 1 à 3, dans laquelle ledit fragment tronqué (b) code pour au moins une toxine polypeptidique active vis-à-vis de Lepidoptera.

5. Cellule selon l'une quelconque des revendications 1 à 4, dans laquelle ledit fragment tronqué (b) est issu d'un ADN qui code pour la protéine cristal Bt2 ou une protéine ayant une séquence substantielle homologue à celle-ci.

6. Cellule selon l'une quelconque des revendications 1 à 5, dans laquelle ledit fragment tronqué (b) est obtenu par troncature d'un ADN qui code pour une protéine cristal produite par un Bt kurstaki, en particulier Bt kurstaki HD1 ou HD73.

7. Cellule selon l'une quelconque des revendications 1 à 5, dans laquelle ledit fragment tronqué (b) est obtenu par troncature d'un ADN qui code pour une protéine cristal produite par un Bt berliner, en particulier Bt berliner 1715.

Patentansprüche

1. Eine transformierte Pflanzenzeile, die ein chimäres Gen enthält, das: stabil in das Genom dieser Zelle integriert ist, in differenzierten Zellen einer Pflanze, die von di ser Zeile abgeleitet ist, exprimiert werden kann und umfaßt:
   (a) eine Promotorregion, abgeleitet von einem Gen, das natürlich in einer Pflanzenzelle exprimiert ist; und
   (b) ein DNA-Fragment, erhalten durch Auslese einer DNA, die für ein Kristallprotein codiert, das von Bacillus thuringiensis erzeugt wird, oder wesentliche Sequenzhomologie dazu aufweist; wobei das ausgelesene Fragment (b) für zumindest ein Polypeptidtoxin des genannten Kristallproteins codiert und eine Insekten-bekämpfende Menge des genannten Polypeptidtoxins in der genannten Zelle liefert als ein Ergebnis von intrazellulärer Expression des genannten ausgelesenen Fragmentes (b).

2. Zelle nach Anspruch 1, worin das ausgelesene Fragment (b) benachbart ist oder verschmolzen ist zu/mit einem auswählbaren oder durchmusterbaren DNA-Markerfragment (c) in dem chimären Gen.

3. Zelle nach Anspruch 2, worin das Markerfragment (c) mit dem ausgelesenen Fragment (b) verschmolzen ist, so daß die Fragmente (b) und (c) ein fusioniertes Polypeptid codieren.

4. Zelle nach einem der Ansprüche 1 bis 3, worin das ausgelesene Fragment (b) für mindestens ein Polypeptidtoxin codiert, das gegen Lepidoptera aktiv ist.

5. Zelle nach einem der Ansprüche 1 bis 4, worin das ausgelesene Fragment (b) von einer DNA stammt, die für das Bt2-Kristallprotein oder für ein Protein mit im wesentlichen Sequenzhomologie dazu codiert.

6. Zelle nach einem der Ansprüche 1 bis 5, worin das ausgelesene Fragment (b) erhalten worden ist durch Auslese einer DNA, die für ein Kristallprotein, produziert von einem Bt Kurstaki, insbesondere Bt Kurstaki HD1 oder HD73, codiert.

7. Zelle nach einem der Ansprüche 1 bis 5, worin das ausgelesene Fragment (b) erhalten worden ist durch Auslese einer DNA, die für ein Kristallprotein, produziert von einem Bt Berliner, insbesondere Bt Berliner 1715, codiert.

8. Zelle nach einem der Ansprüche 1 bis 5, worin das ausgelesene Fragment (b) erhalten worden ist durch Auslese einer DNA, die für ein Kristallprotein, produziert von einem Bt Sotto, codiert.

9. Zelle nach einem der Ansprüche 1 bis 3, worin das ausgelesene Fragment (b) für mindestens ein Polypeptidtoxin codiert, das gegen Coleoptera aktiv ist.

10. Zelle nach einem der Ansprüche 1 bis 9, worin das ausgelesene Fragment (b) ein Polypeptid von 60 bis 80 Kd codiert.

11. Zelle nach Anspruch 10, worin das ausgelesene Fragment (b) erhalten worden ist durch Auslese eines Gens, das ein 130 Kd-Kristallprotein, produziert von einem Bacillus thuringiensis, codiert.

12. Zelle nach Anspruch 11, worin das ausgelesene Fragment (b) die ersten 1820 Nukleotide umfaßt, ausgehend vom N-terminalen Ende des Bt-Gens.

13. Zelle nach einem der Ansprüche 1 bis 11, worin das ausgelesene Fragment ein KpnI-Deletionsfragment einer DNA ist, die für ein Kristallprotein eines Bacillus thuringiensis codiert.

14. Zelle nach einem der Ansprüche 1 bis 13, worin in dem chimären Gen ATG-Triplets zwischen der Promotorregion und einem initiator ATG-Triplet des ausgelesenen Fragmentes (b) fehlen.

15. Zelle nach einem der Ansprüche 1 bis 14, worin das chimäre Gen auch eine 3' nicht-translatierte Region, abgeleitet von einem Gen, das natürlich in einer Pflanzenzelle exprimiert ist, umfaßt.

16. Zelle nach einem der Ansprüche 1 bis 14, worin das chimäre Gen weiter eine 3' nicht-translatierte

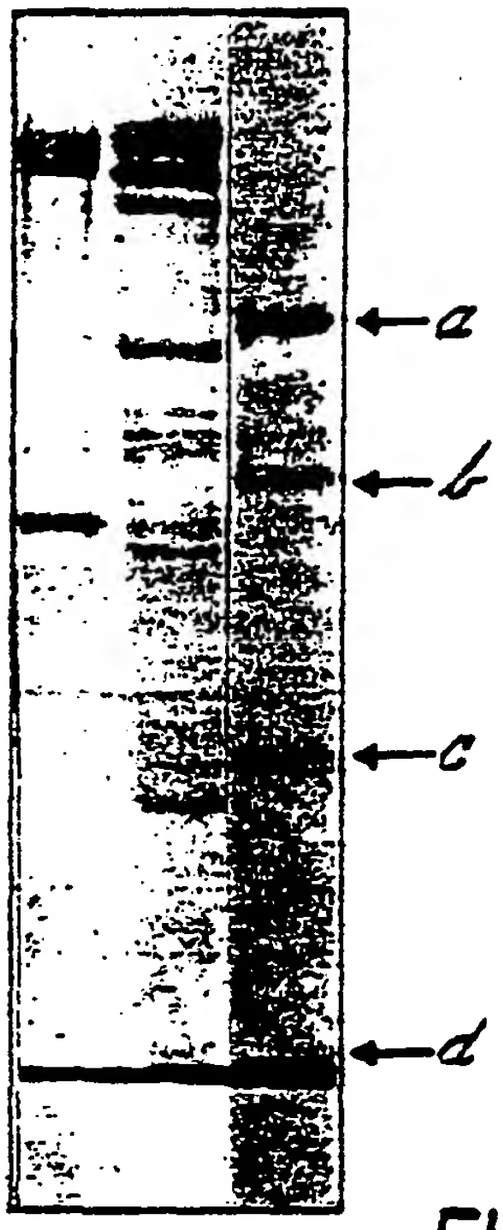

FIG.1.

FIG.2.

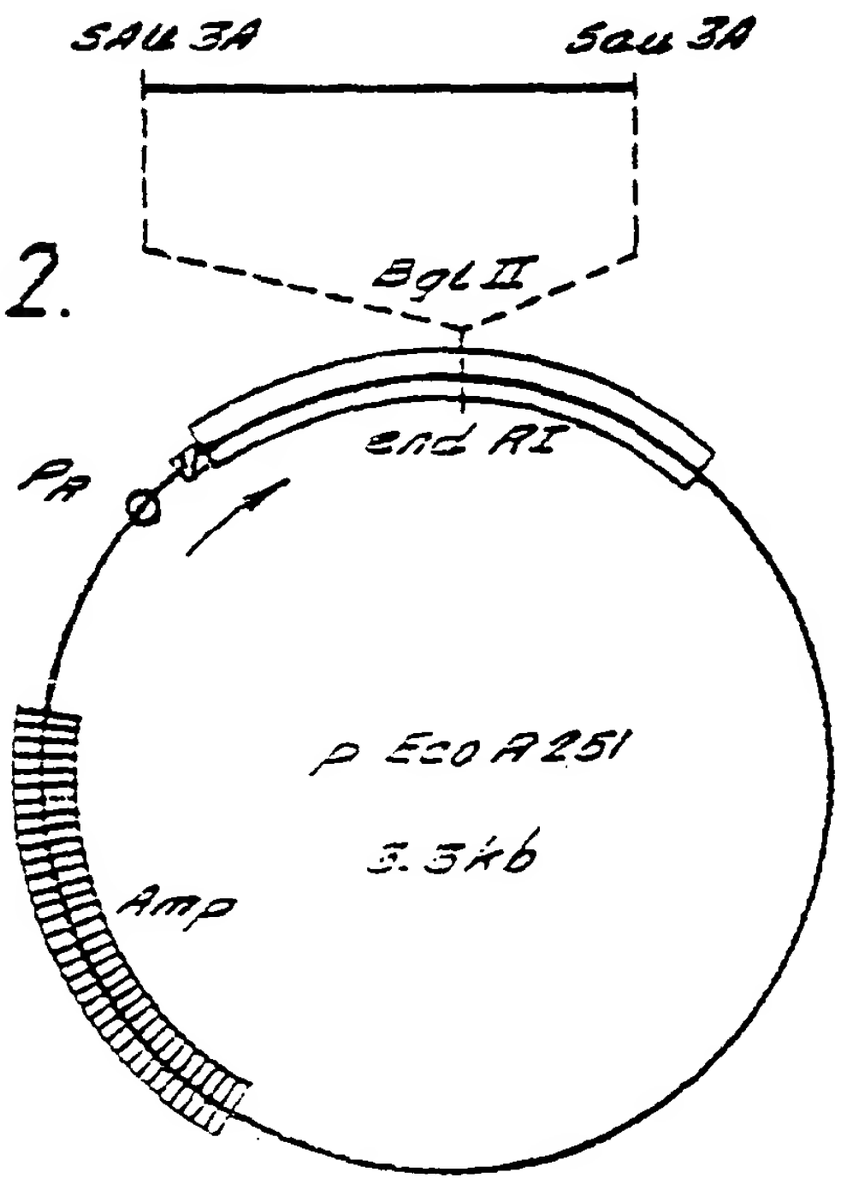

FIG.4.

FIG.5.

FIG.6.

FIG.7.

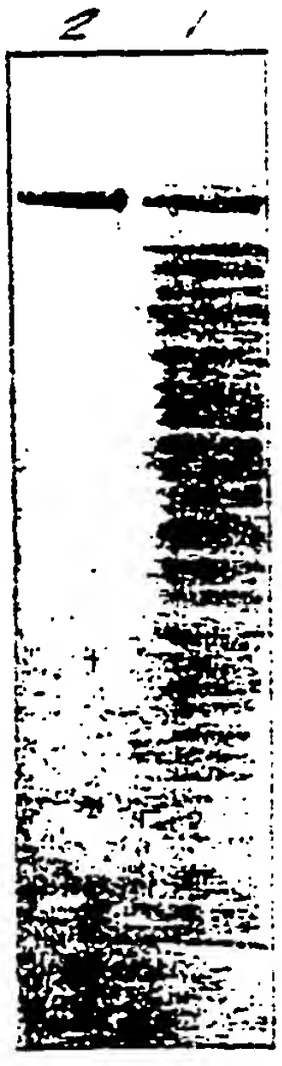

FIG. 10.

COMPARISON OF N-TERMINAL AMINO ACID SEQUENCES OF 130 Kd CRYSTAL PROTEINS

L Bi WHITELEY : Met-Asp-Asn-Asn-Pro-Asn-Ile-Asn-Glu-Cys-Ile-Pro-Tyr-Asn-Cys-Leu-Ser-Asn-Pro

L Bi2 : ?-Asp-Asn-Asn-Pro-Asn-Ile-Asn-Glu-?-Ile-Pro-Tyr-Asn-?-Leu-?-Asn-Pro

FIG. 11.

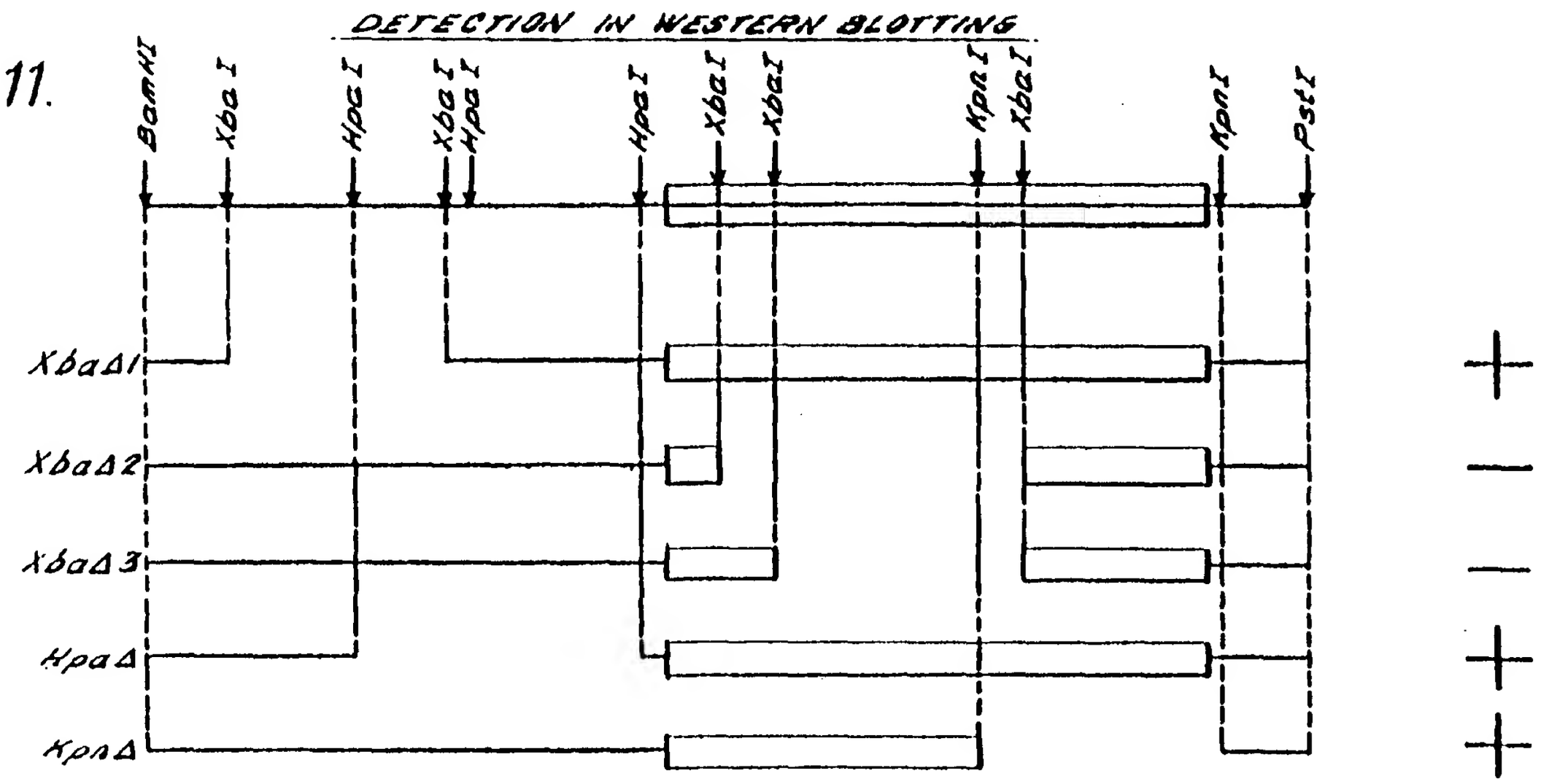

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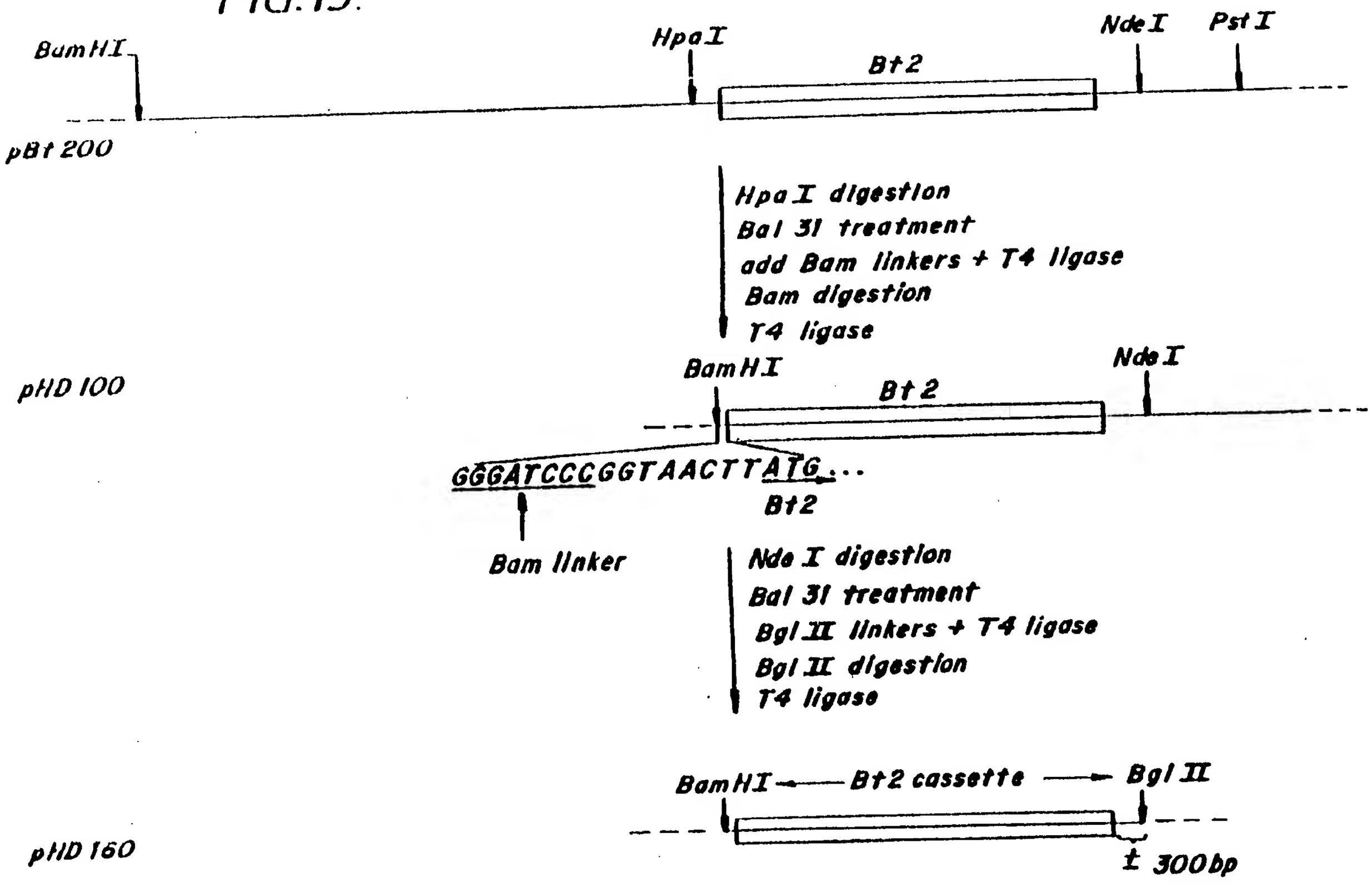
FIG.13A

FIG.14A.

SEQUENCE COMPARISON OF FOUR BACILLUS THURINGIENSIS TOXINS

```
          10                  20                  30                  40                  50
MDNNPNINEC  IPYNCLSNPE  VEVLGGERIE TGYTPIDISL  SLTQFLLSEF

          60                  70                  80                  90                 100
VPGAGFVLGL  VDIIWGIFGP  SQWDAFLVQI EQLINQRIEE  FARNQAISRL
                                    P

         110                 120                 130                 140                 150
EGLSNLYQIY  AESFREWEAD  PTNPALREEM RIQFNDMNSA  LTTAIPLFAV
                                                    .   L

         160                 170                 180                 190                 200
QNYQVPLLSV  YVQAANLMLS  VLRDVSVFGQ RWGFDAATIN  SRYNDLTRLI

         210                 220                 230                 240                 250
GNYTDHAVRW  YMTGLERVWG  PDSRDWIRYN QFRRELTLTV  LDIVSLFPNY
       Y                            V                      A
       Y                            V                      A   S
       Y                            V                      A   S

         260                 270                 280                 290                 300
DSRTYPIRTV  SQLTREIYTN  PVLENFDGSF RGSAQGIEGS  IRSPHLMDIL
    R                                                  R
    R                                M   R   QN     Q
    R                H               M   R   QN     Q

         310                 320                 330                 340                 350
NSITIYTDAH  RGEYYWSGHQ  IMASPVGFSG PEFTFPLYGT  MGNAAPQQRI
                  Y
         V      FN          T                  A   F N  A       PV-L
 R       V      FN          T                  A  VF N  A       PV-L

         360                 369                 379                 389                 398
VAQLGQGVYR  TLSSTLYRR-  PFNIGINNQQ LSVLDGTEFA  YGTSS-NLPS
 SLT  L IF       P     I  ILGS P   E  F        S  FASLTT
 SLT  L IF       P     I  ILGS P   E  F        S  FASLTT

         408                 418                 428                 438                 448
AVYRKSGTVD  SLDEIPPQNN  NVPPRQGFSH RLSHVSMFRS  GFSNSSVSII

TI   QR         V    D   S    A              T LSQ  AAGAVYTL--
TI   QR         V    D   S    A              T LSQ  AAGAVYTL--

         458                 468                 478                 488                 498
RAPMFSWIHR  SAEFNNIIPS  SQITQIPLTK STNLGSGTSV  VKGPGFTGGD
                        A   DS      AV  GNF FN -      IS
                                                              H
    T    Q
    T    Q

         508                 515                 523                 533                 543
ILRRTSPGQI  STLRVNI---  -TAPL-SQRY RVRIRYASTT  NLQFHTSIDG
LV LN S NN  IQN GY EVP  IHF ST T      V     V  PIHLMVNWGN

         553                 563                 573                 583                 593
RPINQGNFSA  TMSSGSNLQS  GSFRTVGFTT PFNFSNGSSV  FTLSAHVFNS
SS FSNTVP    AT LD      SQ---F YFE SA AFTS LG  NIVGVRN SG
```

FIG.15.



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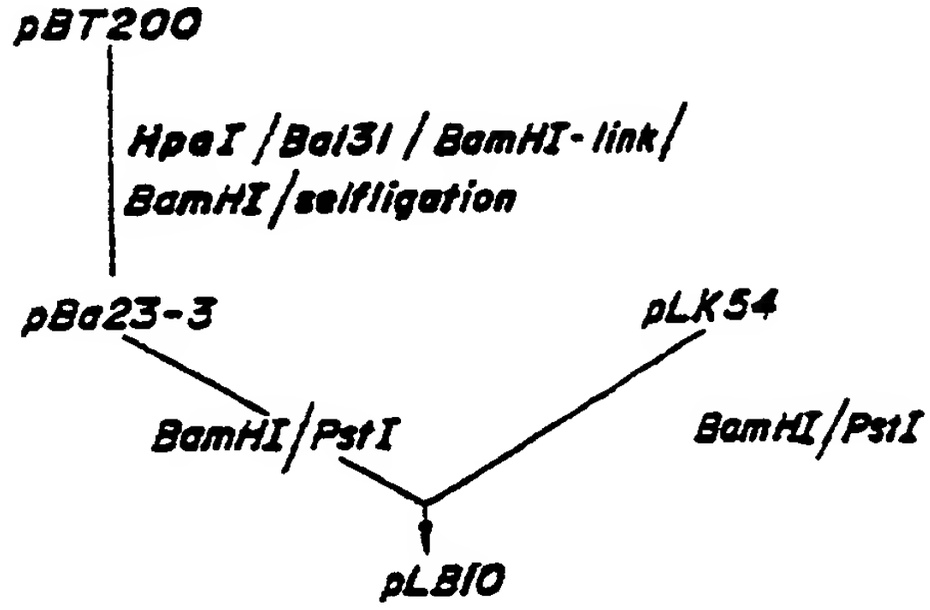
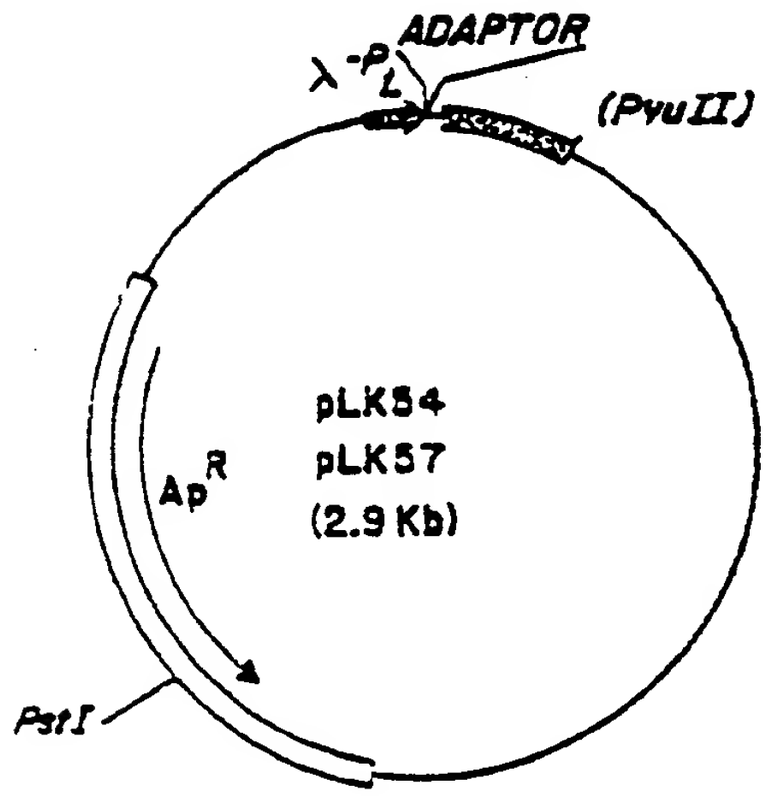
STRATEGY USED FOR THE POSITIONING OF THE TOXIN GENE BEHIND THE λ-P_L PROMOTOR.

FIG.17.





PLASMID	ADAPTOR
pLK54	AATTCCCGGGGATCCGTCGACCTGCAGCCAAGCTTGGTCTAGAGGTCGA EcoRI SmaI BamHI SalI PstI HindIII XbaI
pLK57	AATTCCCSGGAGAGCTCGATATCGCATGCGGTACCTCTAGAAG EcoRI SmaI SacI EcoRV SphI KpnI XbaI AAGCTTGGGATCCGTCGACCTGCAGATCTGCTAGAGGTCGA HindIII BamHI SalI PstI BglII

FIG. 19.

Construction of random Bt2:NPTII fusions and Bt2 deletions

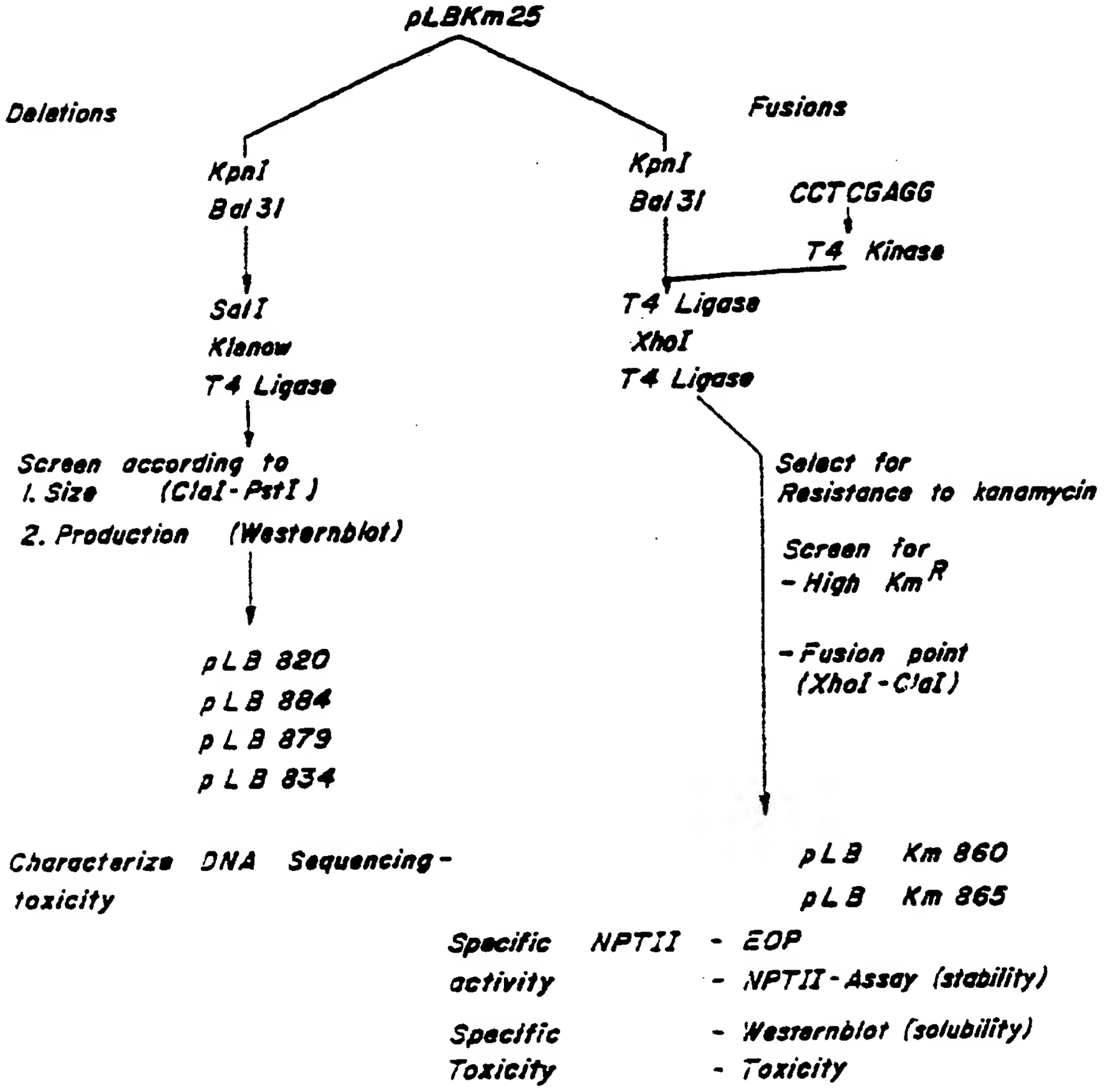
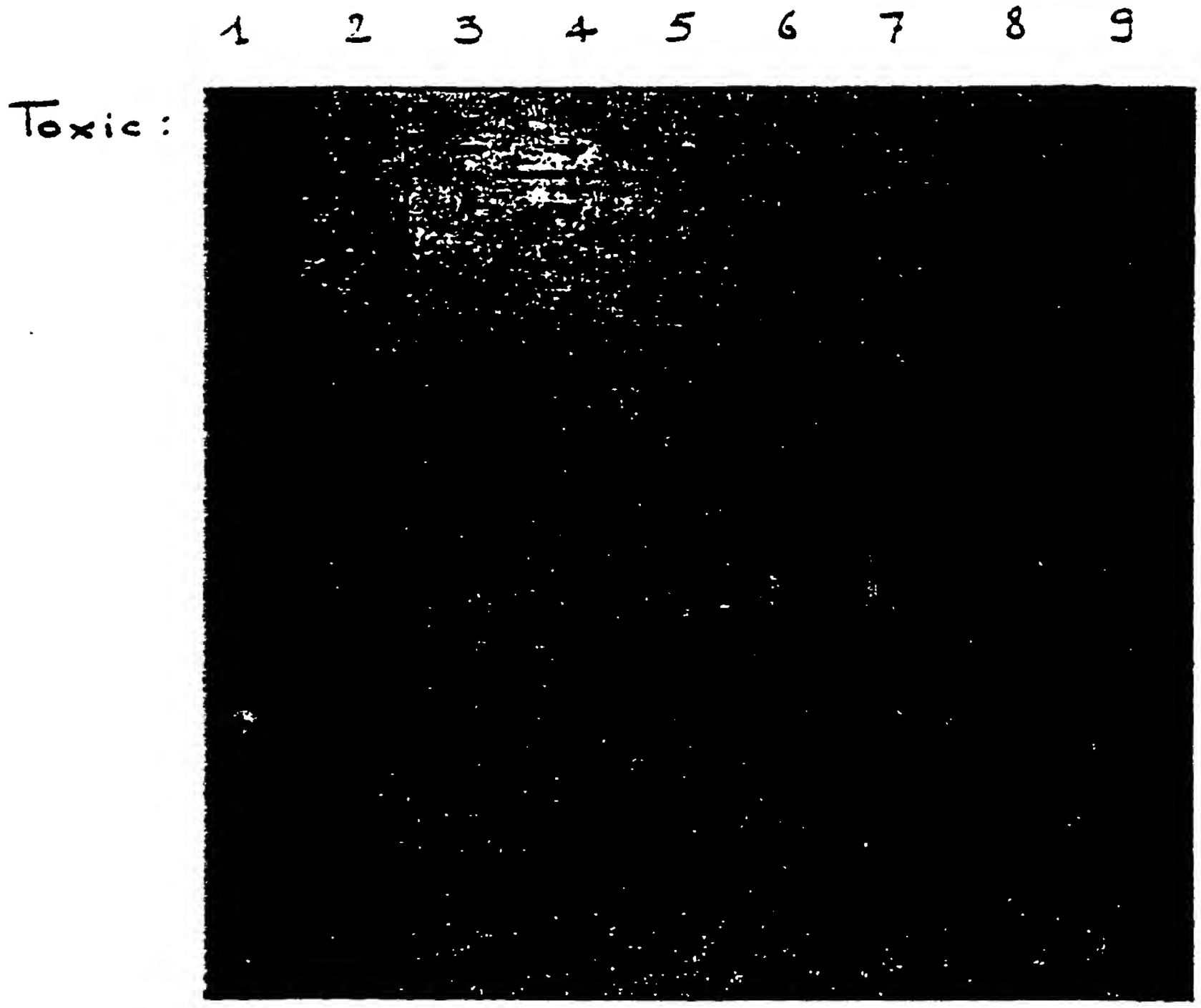


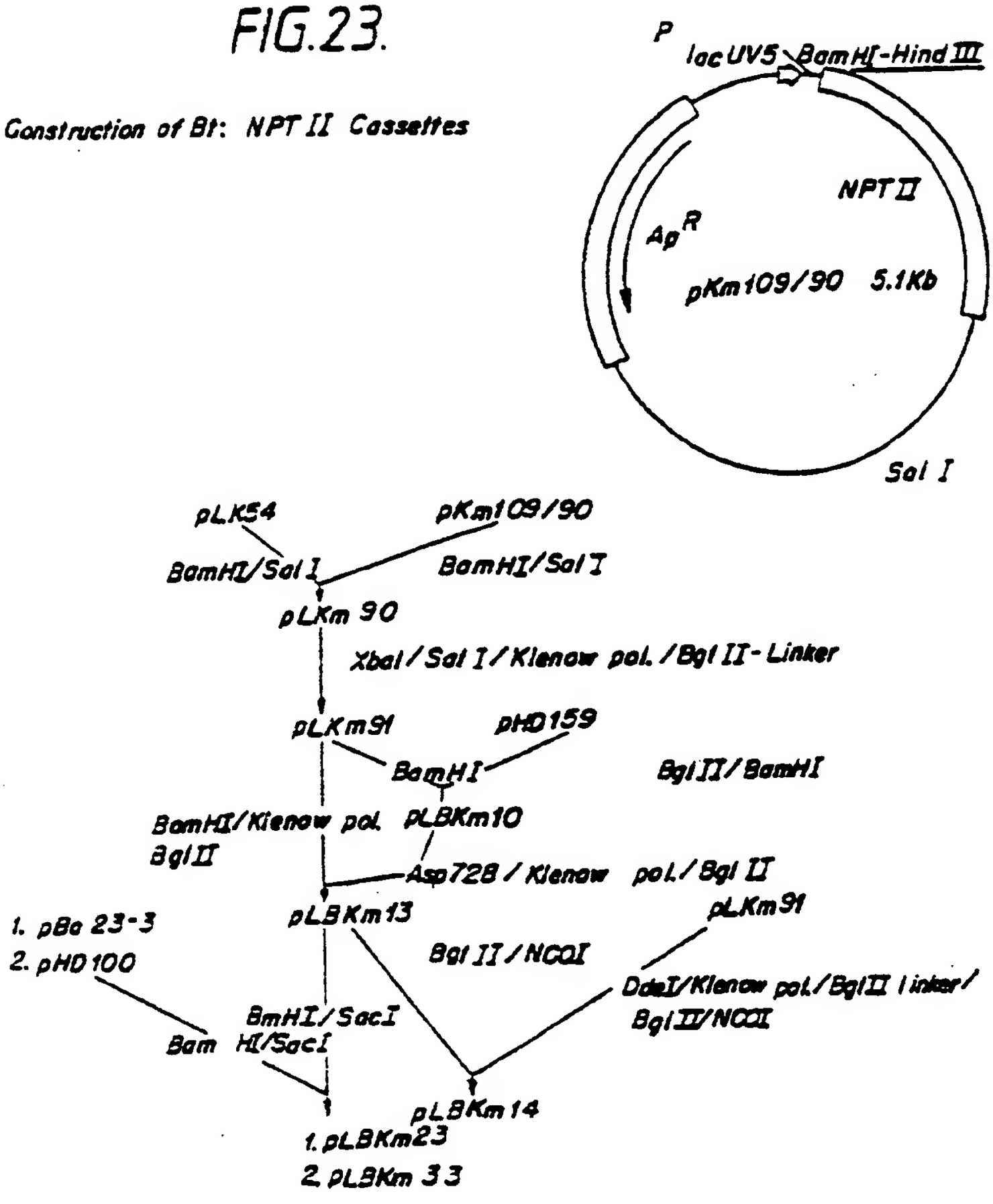
FIG.21.



1. Trypsin digest Bt2
2. pLB 950
3. pLB 334
4. pLB 379
5. pLB 884
6. pLB 828
7. pLB 886
8. NF1 control

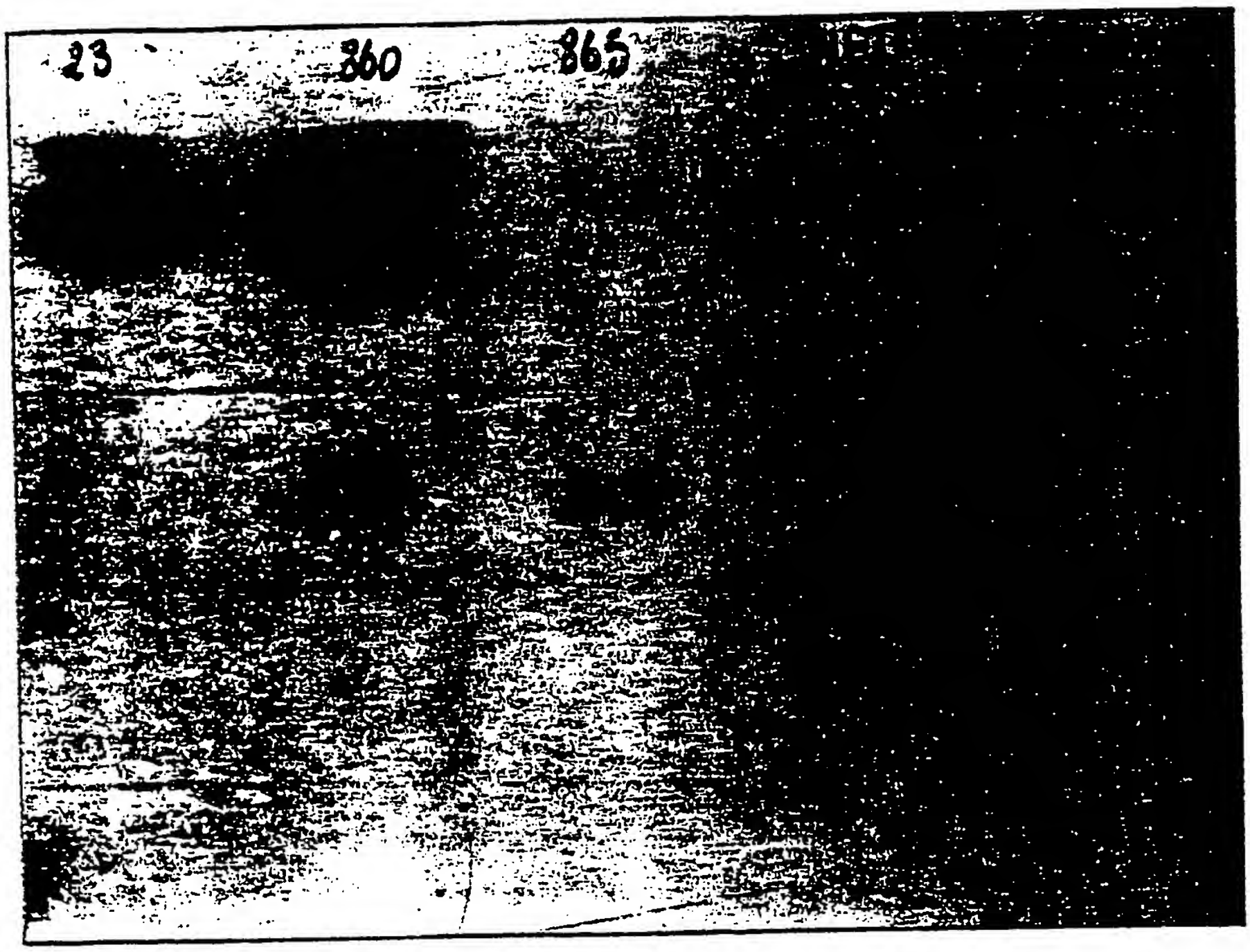
FIG.23.

Construction of Bt: NPT II Cassettes



Plasmid	5' ends of the Bt2 gene	Purpose
pLBKm 13	GGAT'CCC'GAT... +4 Bt2	Fusion at initiator ATG
pLBKm 23	GGATCCCGTGGTATCTTAATTAAAAGAGATG GAGGTAACTT'ATG'GAT ... +1 Bt2	Expression in E. coli
pLBKm 33	GGATCCCGTAACTT'ATG'GAT... BamHI +1 Bt2	Fusion to plant promotor

FIG. 25.



23 = B+:NPT2

860 = B+:NPT860

865 = B+:NPT865

NPT = NPT II intact

NF1 = control extract

FIG.27.

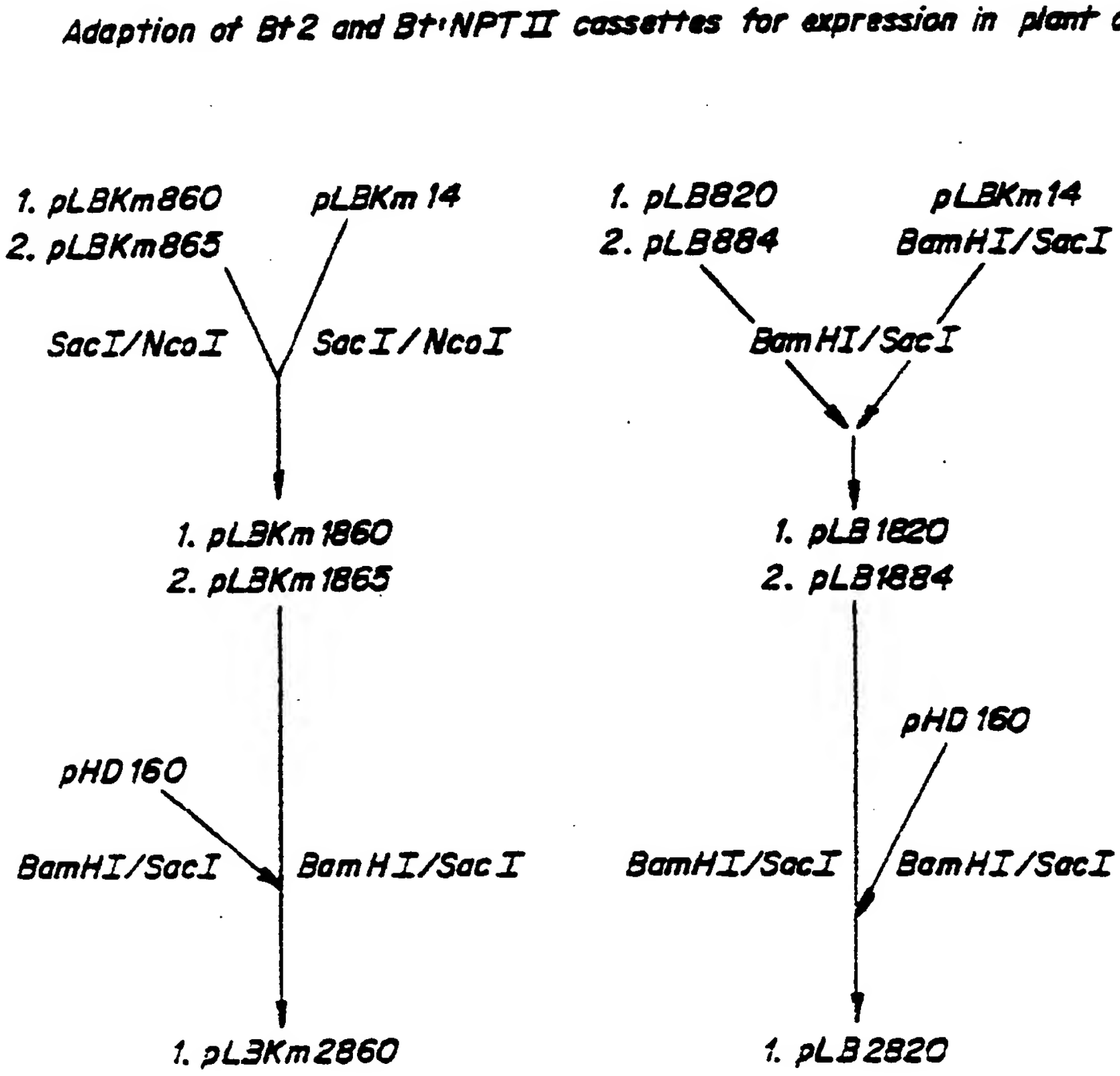
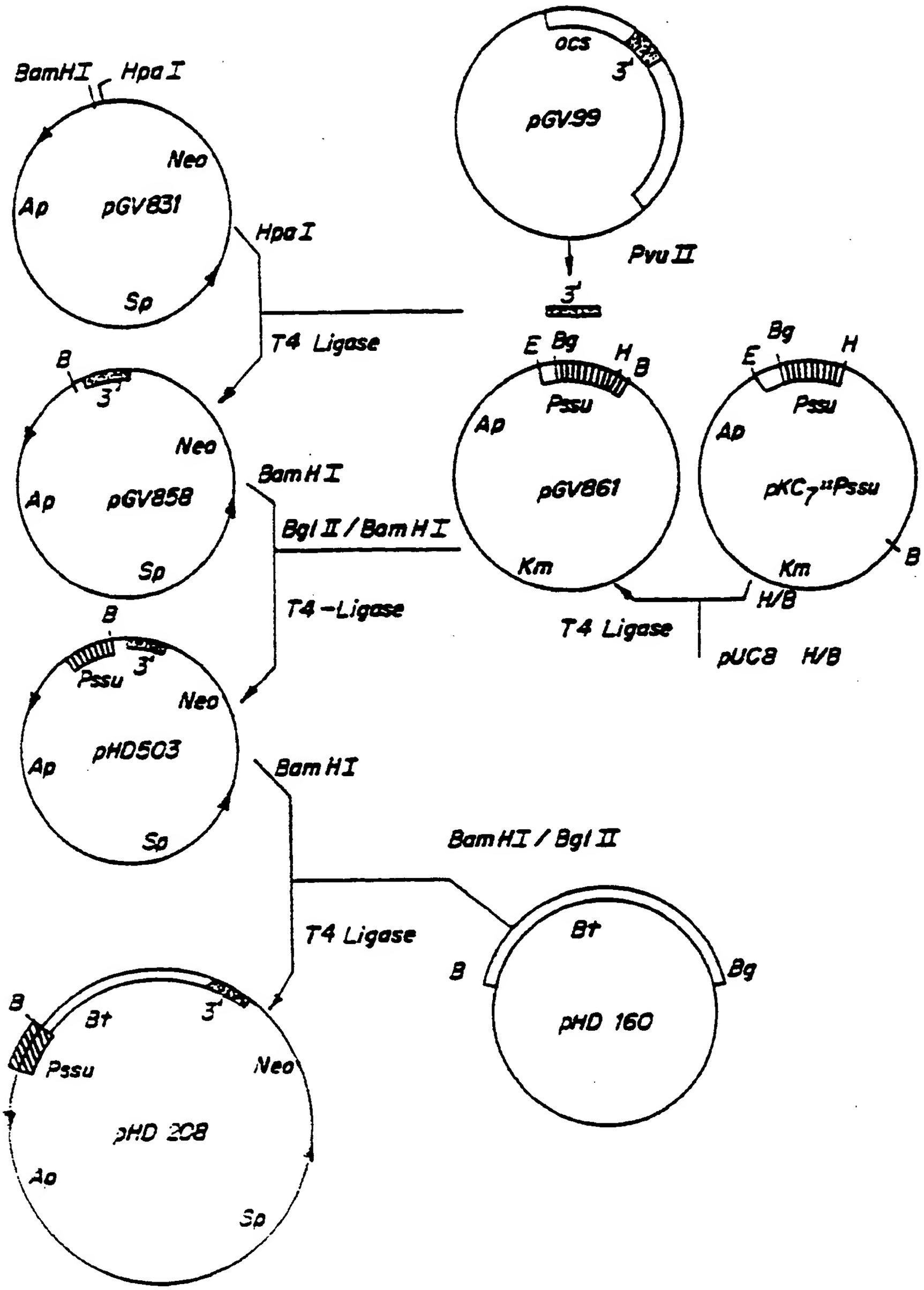

Adaption of Bt2 and Bt·NPTII cassettes for expression in plant cells·
1. pLBKm860
2. pLBKm865
pLBKm 14
SacI/NcoI
SacI/NcoI
1. pLBKm1860
2. pLBKm1865
pHD160
BamHI/SacI
BamHI/SacI
1. pL3Km2860
1. pLB820
2. pLB884
pLBKm14
BamHI/SacI
BamHI/SacI
1. pLB1820
2. pLB1884
pHD160
BamHI/SacI
BamHI/SacI
1. pLB2820

FIG.29.


BamHI
HpaI
Neo
Ap
pGV831
Sp
HpaI
T4 Ligase
OCS
3'
pGV99
PvuII
3'
B
3'
Neo
Ap
pGV858
Sp
BamHI
Bgl II / BamHI
T4 -Ligase
E
Bg
H
B
Pssu
Ap
pGV861
Km
Bg
H
E
Pssu
Ap
pKC7"Pssu
Km
B
H/B
T4 Ligase
pUC8 H/B
B
3'
Pssu
Neo
Ap
pHD503
Sp
BamHI
BamHI / Bgl II
T4 Ligase
Bt
B
Bg
pHD 160
B
Bt
3'
Pssu
Neo
pHD 208
Ap
Sp

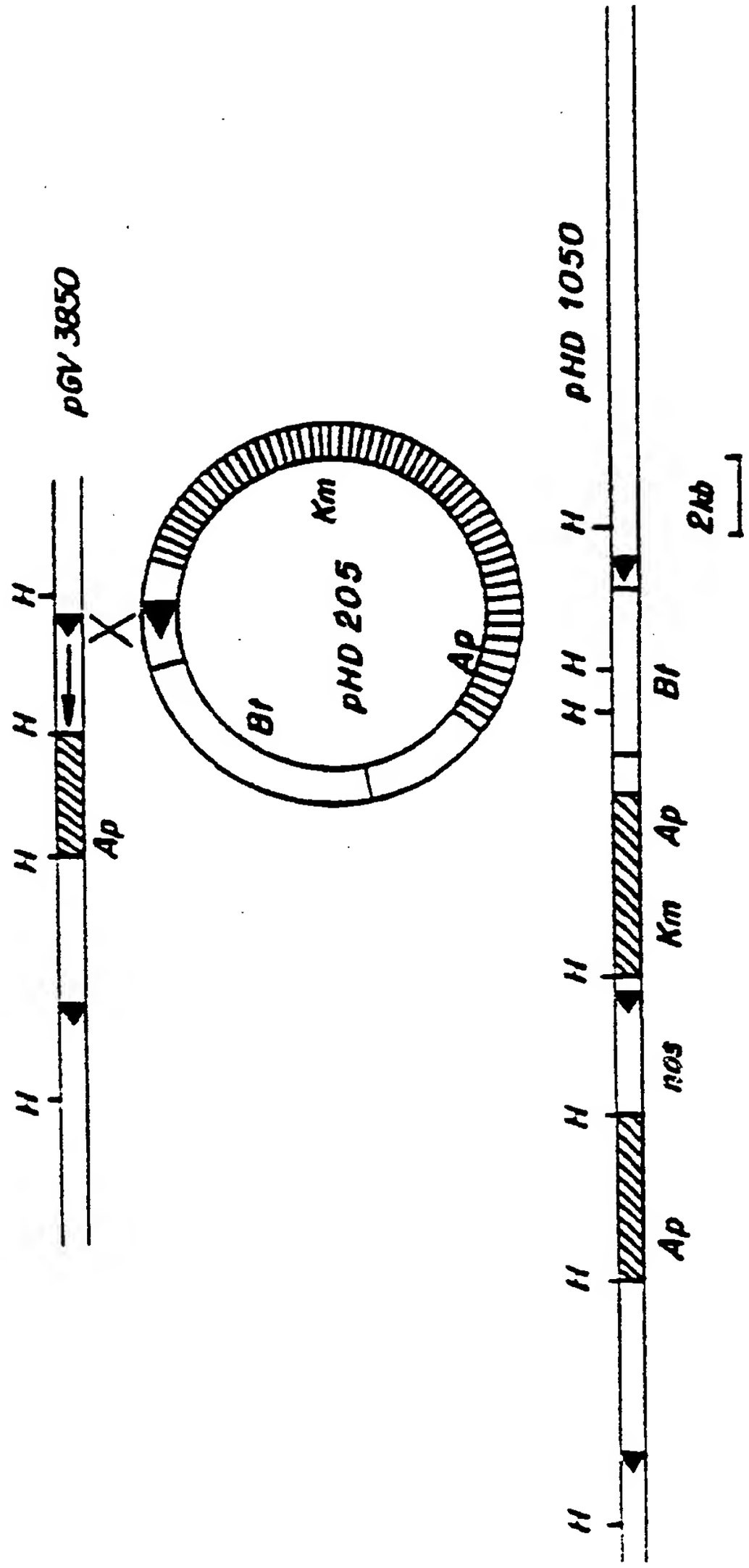
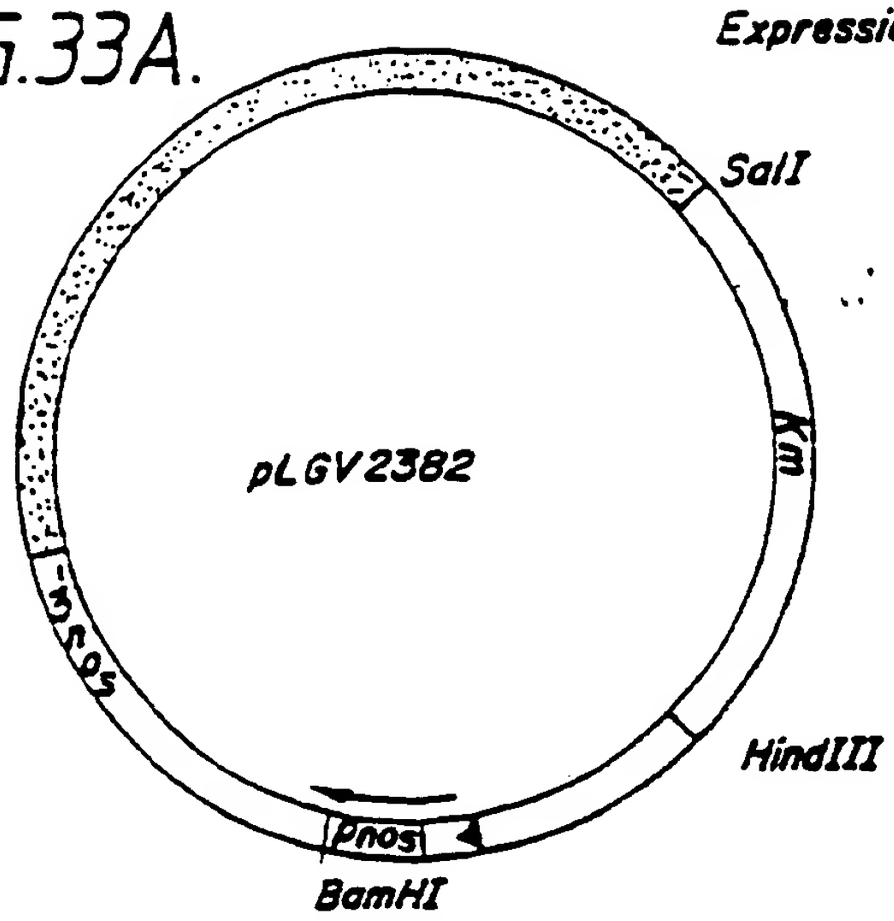

pGV 3850
H
H
H
H
Ap
X
Bl
pHD 205
Km
Ap
pHD 1050
H
H
H H
Bl
H
H
Km
Ap
nos
Ap
H
H
2kb

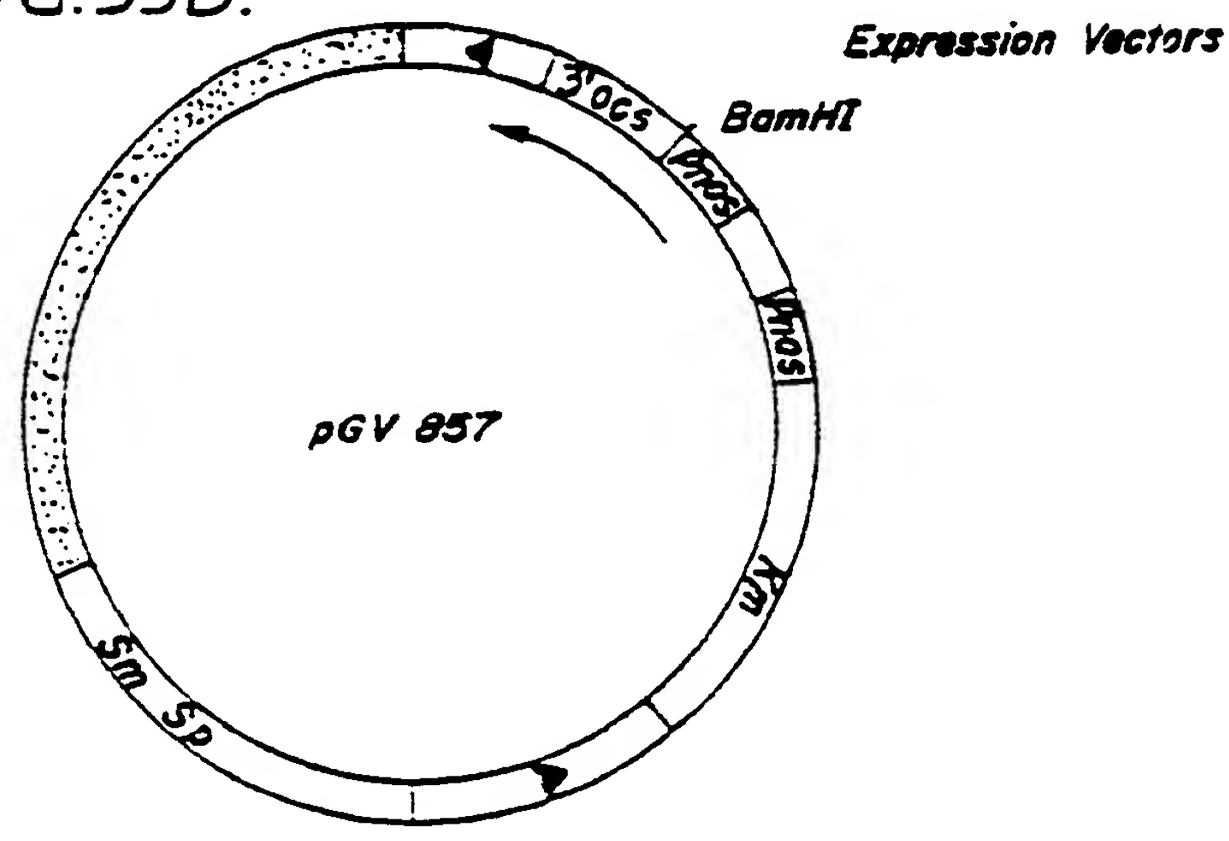
FIG. 31.

FIG.33A.



pLGV2382 : Herrera-Estrella et al, (1983) EMBOJ, 2, 987

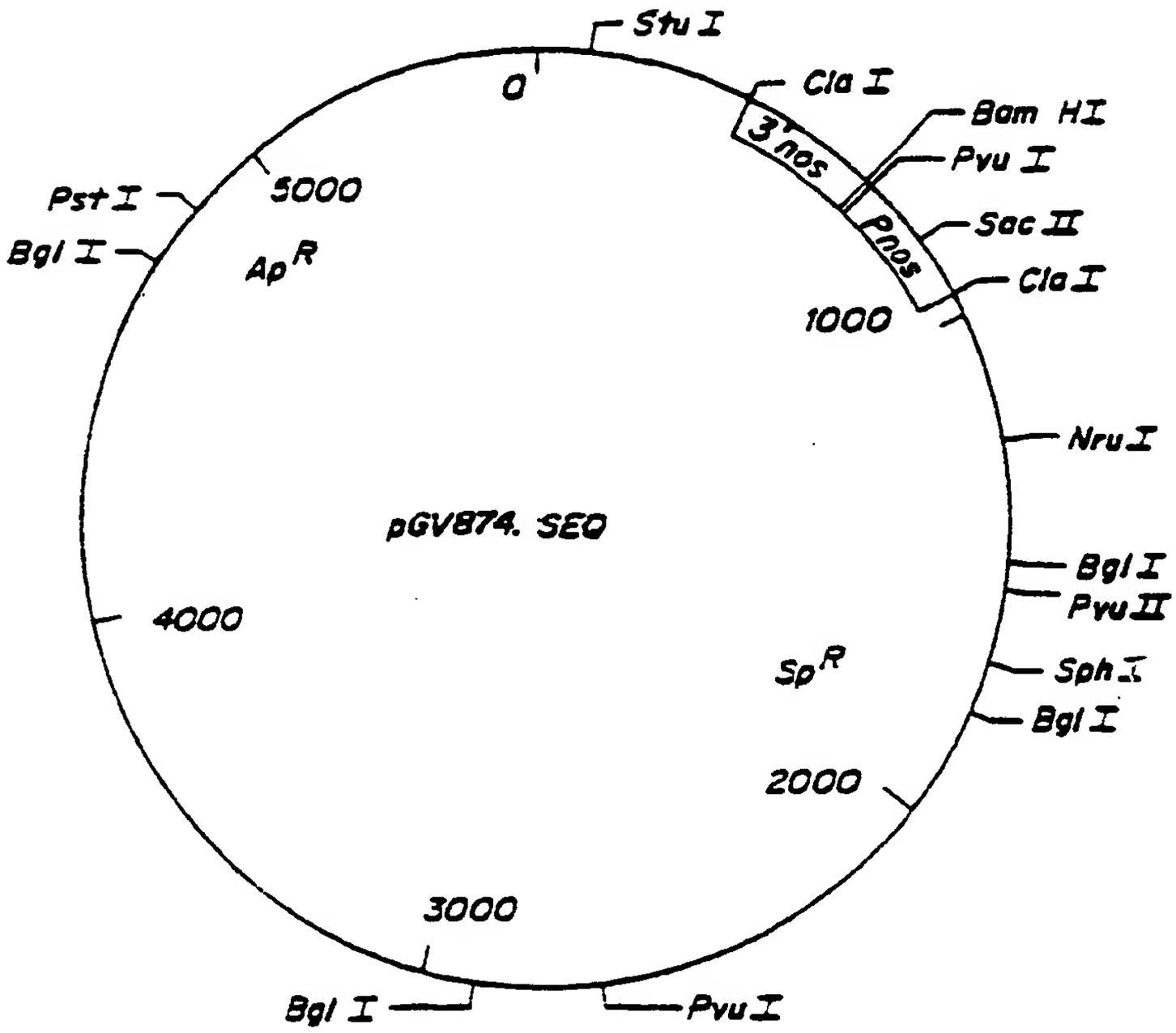
FIG.33B.



derived from pGV831 (DeBlaere et al, 1985)
Pnos : bp BamHI-BclI fragment of pLGV23
3'ocs : 706 bp PVUII fragment of ocs in HpaI site of pGV831
◀ : T-DNA Border

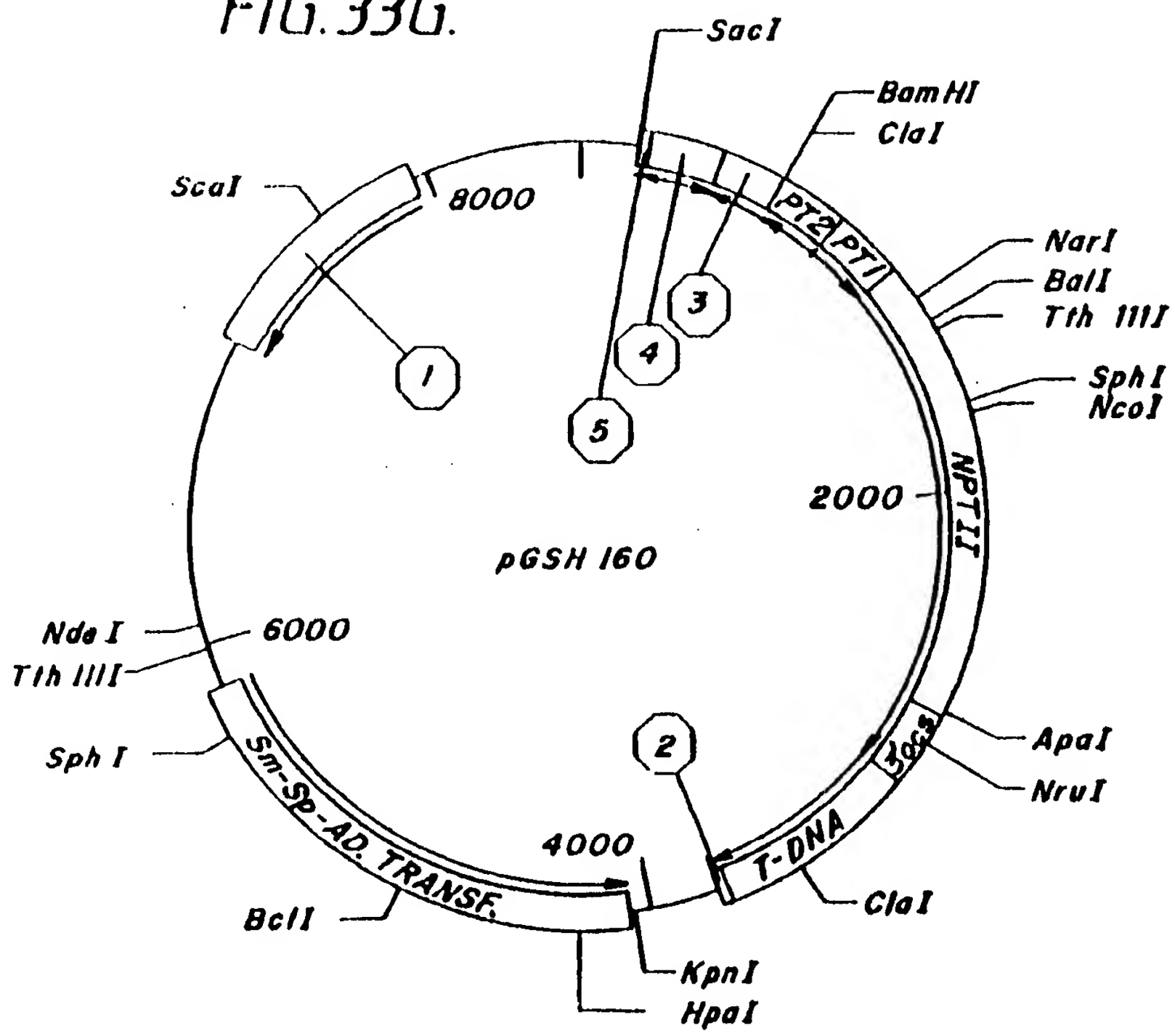
FIG.33E.

Expression vector pGV874:



- "Pnos" fragment is the BclI-Bam HI fragment from pLGV2382 (Herrera-Estrella et al., EMBO J., 2, 987, 1983).
- "3' nos" fragment is the 687 bp TaQ1-Hind III fragments from the nos gene. (De Picker et al., J.M.A.G., 1, 561, 1982).
- "Sm-Sp" is the 2.3 Kb Hind III / Bam HI fragment from R702.
- The remaining of the vector is pBR322.

FIG. 33G.



PLANT EXP. VECTOR

LEGEND

Sequence from embl. databank
Sequence from 1 to 8558
Sites from 1 to 8558
Maximal occurence frequency : 2
All enzymes from ENZ. GOOD

1. BETA-LACTAMASE
2. L. B.
3. 3' END T7
4. T-DNA
5. R. B.

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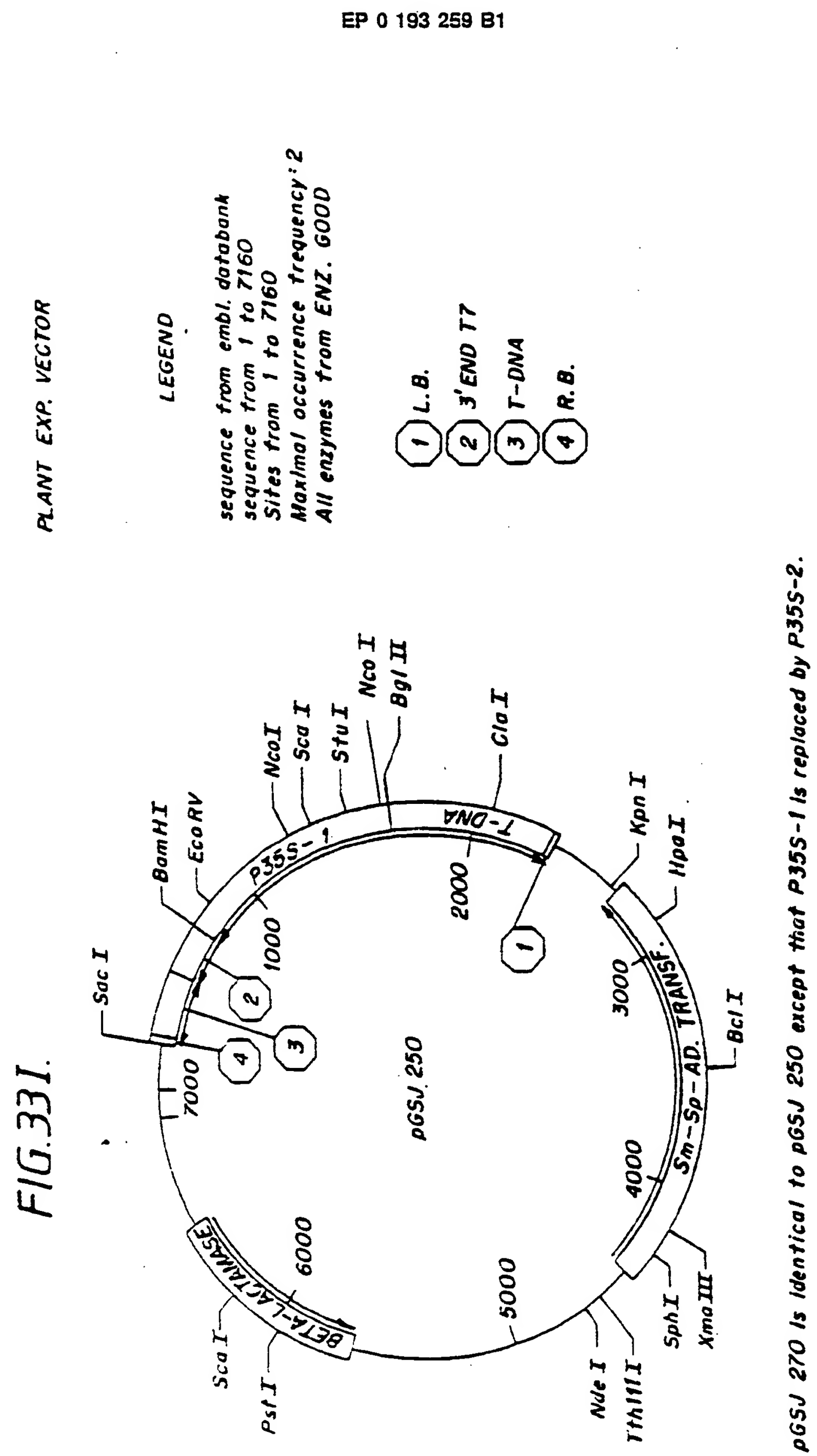
FIG.33I.



pGSJ 270 is identical to pGSJ 250 except that P35S-1 is replaced by P35S-2.

FIG.34.

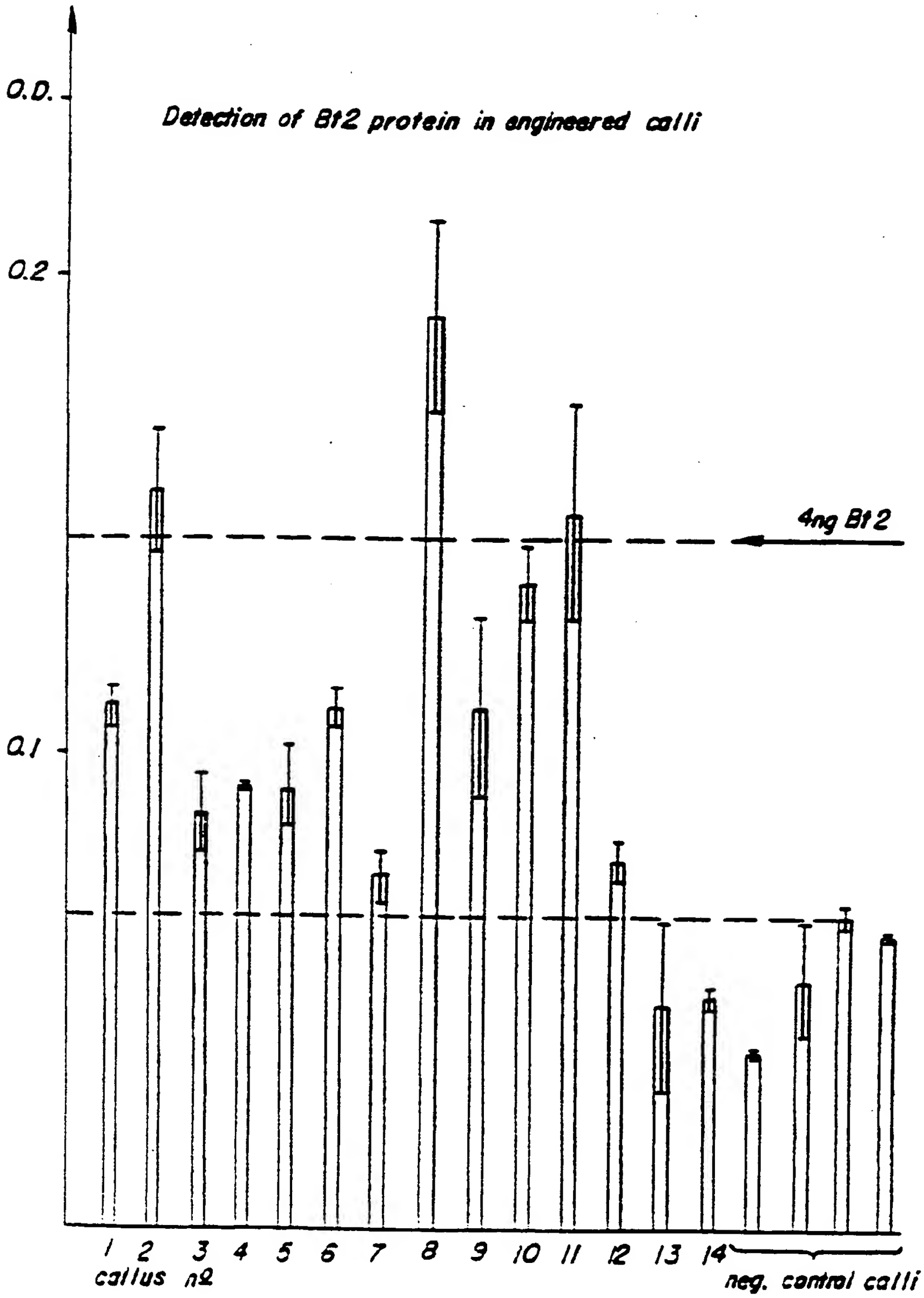

O.D.
Detection of B12 protein in engineered calli
0.2
0.1
4ng B12
1 2 3 4 5 6 7 8 9 10 11 12 13 14
callus nº
neg. control calli

FIG.36.

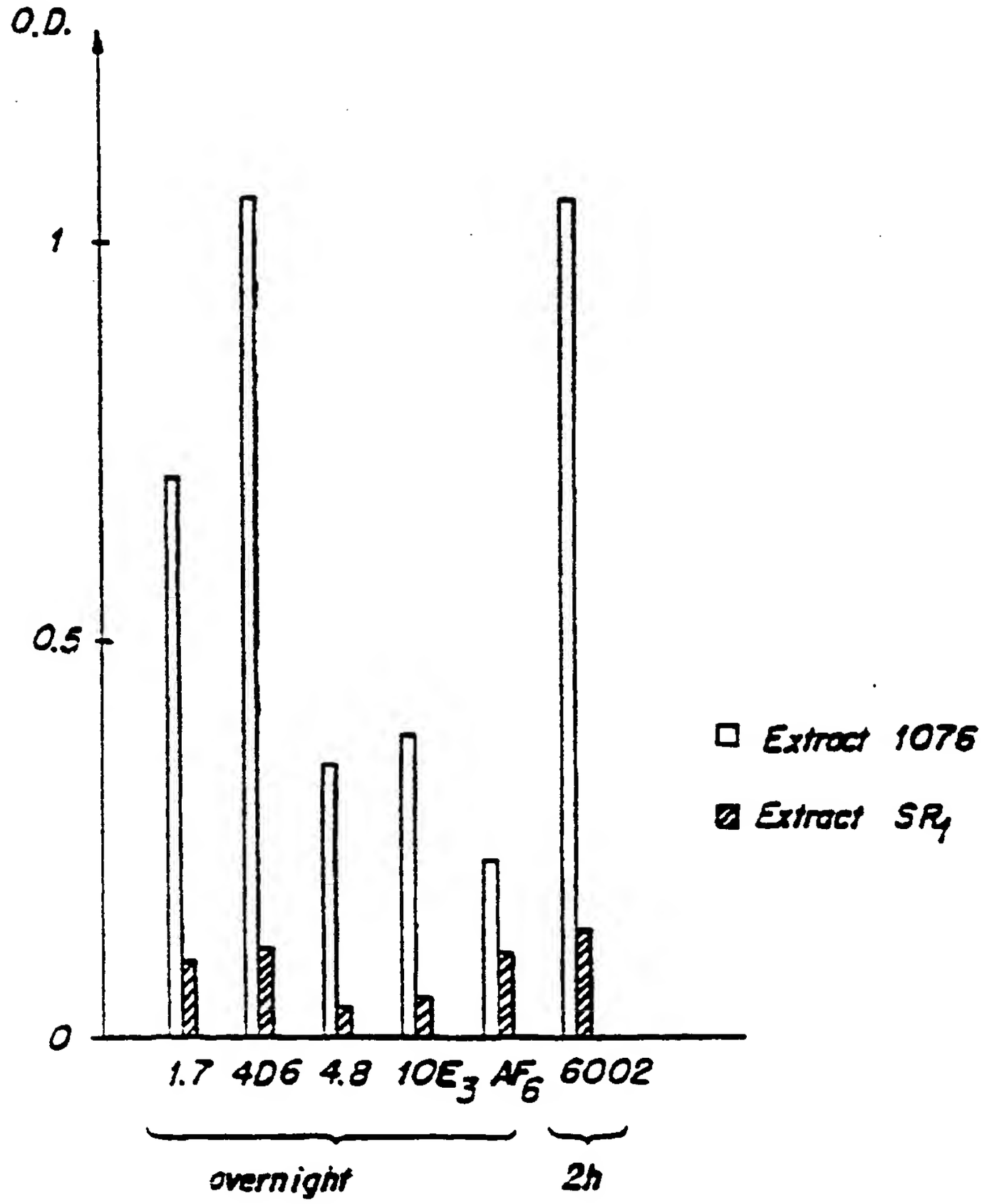

O.D.
1
0.5
0
1.7
406
4.8
10E3
AF6
6002
overnight
2h
Extract 1076
Extract SR1

FIG. 37B.

Supernatant II:

- Acid precipitation: bring pH down slowly to 4.5 by adding dropwise 1 M HCl
- Incubate for 30 min at 0°C
- Centrifuge 10,000 rpm, 30min
- Wash once with cold distilled H_2O
- Resuspend pellet in small volume of buffer: Na_2CO_3 pH 10 50 mM DTT 5 mM PMFS 0.17mg/ml
- Incubate for 1 h at 0°C, while regularly resuspending
- Centrifuge (in Eppendorf)

supernatant = fraction II

Pellet II:

- Resuspend in 25 ml extraction buffer containing:
  2 % SDS
  Na_2CO_3 pH 10 100mM
  DTT 10 mM
  and agitate for 15 min
- Centrifuge 13,000 rpm, 30 min
- Supernatant —— acetone precipitation:
  mix with 9 volumes of acetone 1/40 vol 1 MHCl
- Incubate overnight at −20°C
- Centrifuge 13,000 rpm, 20 min
- Resuspend pellet in small volume of buffer containing { 2% SDS; Na_2CO_3 pH10 100mM; DTT 10mM }
  and boil for 10 min
- Centrifuge —— sup = fraction III

FIG.39.

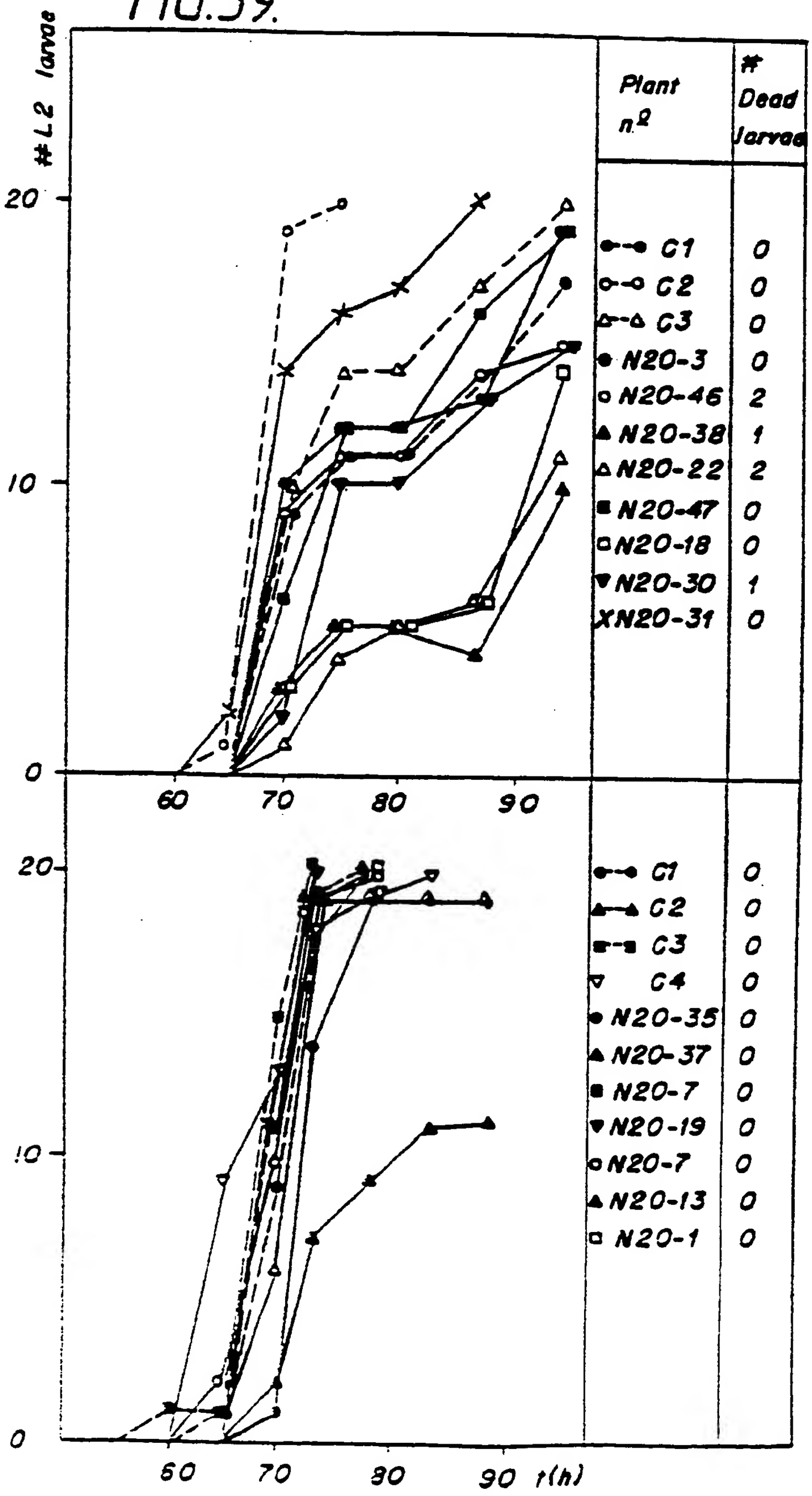

#L2 larvae
20
10
0
60
70
80
90
Plant n.º
Dead larvae
C1 0
C2 0
C3 0
N20-3 0
N20-46 2
N20-38 1
N20-22 2
N20-47 0
N20-18 0
N20-30 1
XN20-31 0
20
10
0
60
70
80
90
t(h)
C1 0
C2 0
C3 0
C4 0
N20-35 0
N20-37 0
N20-7 0
N20-19 0
N20-7 0
N20-13 0
N20-1 0

FIG.41.

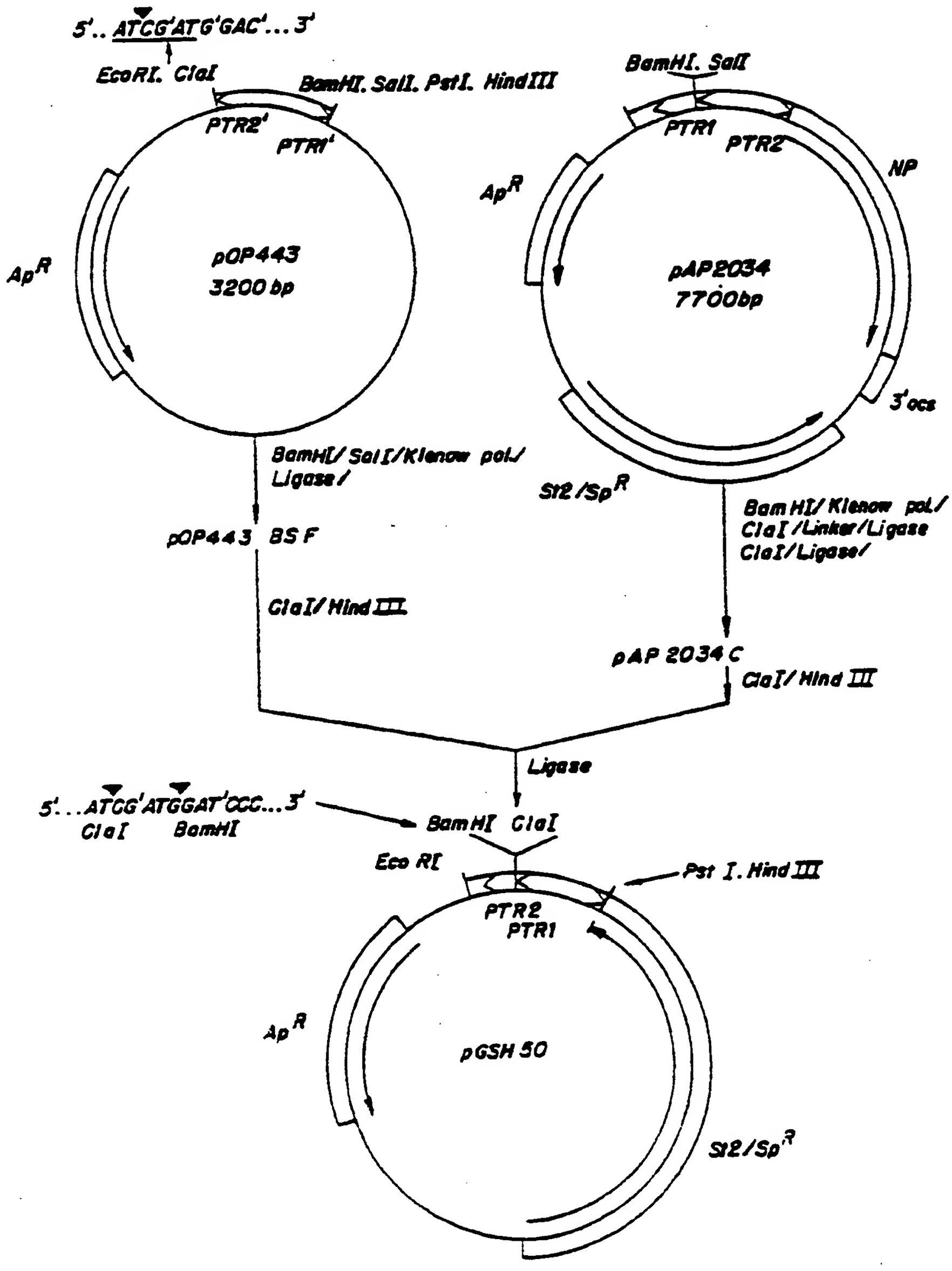
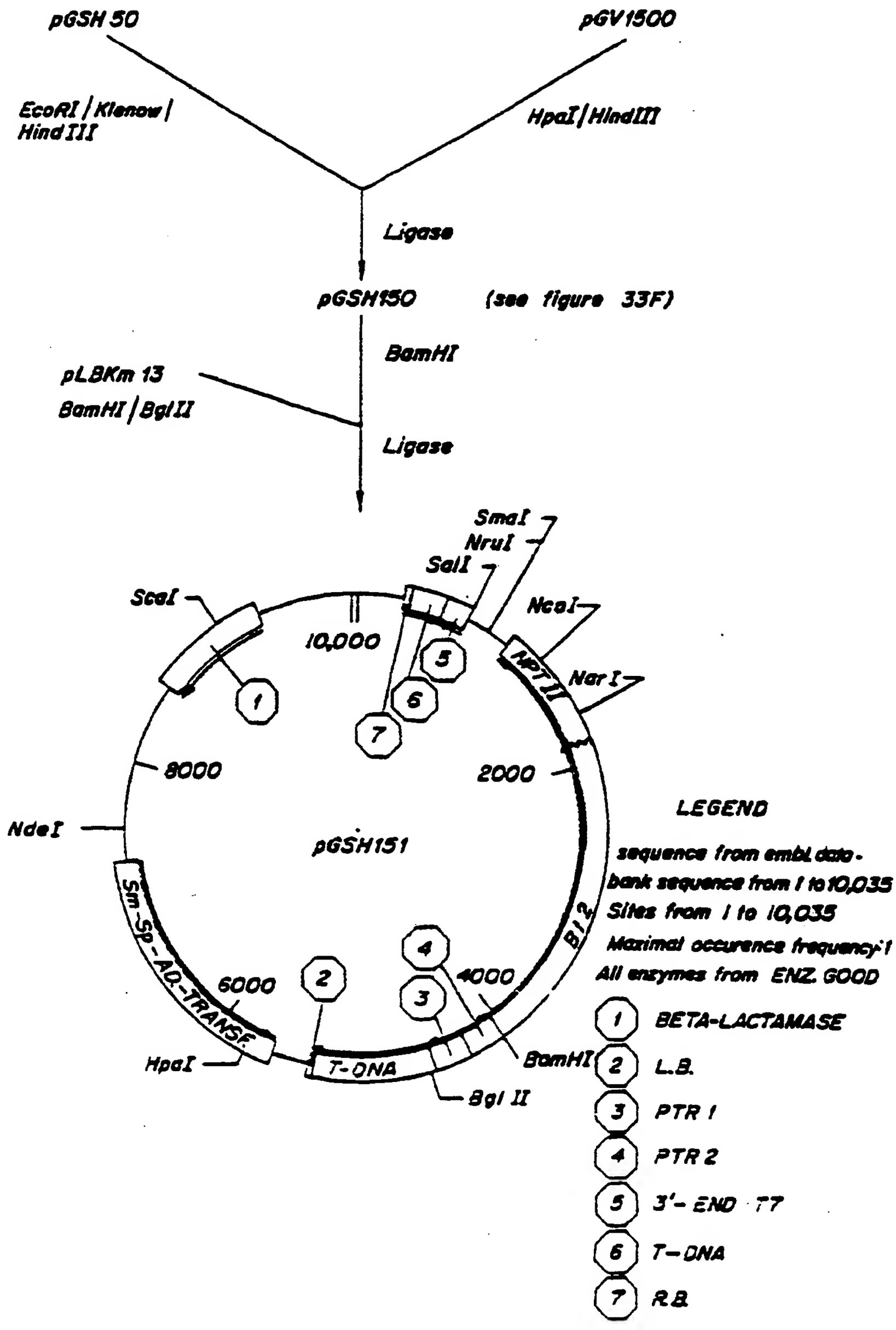

5'.. ATCG'ATG'GAC'... 3'
EcoRI. ClaI
BamHI. SalI. PstI. HindIII
PTR2'
PTR1'
ApR
pOP443
3200 bp
BamHI. SalI
PTR1
PTR2
NP
ApR
pAP2034
7700bp
3'ocs
BamHI/ SalI/Klenow pol/ Ligase/
St2 /SpR
BamHI/ Klenow pol/ ClaI /Linker/Ligase ClaI/Ligase/
pOP443 BS F
ClaI/ Hind III
pAP 2034 C
ClaI/ Hind III
Ligase
5'...ATCG'ATGGAT'CCC...3'
ClaI BamHI
BamHI ClaI
Eco RI
Pst I. Hind III
PTR2
PTR1
ApR
pGSH 50
St2/SpR

FIG.43


pGSH 50
pGV1500
EcoRI / Klenow / HindIII
HpaI / HindIII
Ligase
pGSH150
(see figure 33F)
BamHI
pLBKm 13
BamHI / BglII
Ligase
SmaI
NruI
SalI
ScaI
NcoI
10,000
NarI
8000
2000
NdeI
pGSH151
Sm-Sp-AD-TRANSF.
B12
6000
4000
HpaI
T-DNA
BamHI
Bgl II
LEGEND
sequence from embl data.
bank sequence from 1 to 10,035
Sites from 1 to 10,035
Maximal occurence frequency: 1
All enzymes from ENZ. GOOD
1 BETA-LACTAMASE
2 L.B.
3 PTR 1
4 PTR 2
5 3'- END T7
6 T-DNA
7 R.B.